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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)* Estrogen binds specific receptors present in 70% of breast cancers. Estrogen receptor (ER) is a phosphoprotein that regulates transcription and growth by binding estrogen response elements (ERE) in DNA. Inactive ER is a monomer that forms dimers on estrogen-induced phosphorylation ER transcriptional activity is regulated by distinct conformational states resulting from ligand binding, and the induced complex recruits steroid receptor coactivator proteins, such as SRC-1, that are essential for growth. Peptides modeled from interacting sites of ER may selectively inhibit ER signals and act as antiestrogens. To test this hypothesis, we made small peptides to mimic highly conserved ER sequence at tyrosine-537 and surrounding leucine residues. Peptide antiestrogens, but not control peptides, block ER association with SRC-1 and disrupt binding of ER to ERE. In *in vitro* studies, estradiol stimulates breast cell growth, and this estrogen effect is blocked by peptide antiestrogens conjugated with *Antennapedia* carrier, but not controls. Using *in vivo* tumor xenografts, treatment with peptide antiestrogens shows significant activity in arresting growth of estrogen-dependent breast tumors. This work provides target validation but also shows that peptide drugs are difficult to administer. Thus, we have prepared more lipid-like, peptidomimetric derivatives that function similarly but may be easier to use in the clinic.

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INTRODUCTION

The estrogen receptor (ER) is a phosphoprotein found in 70% of human breast tumors at diagnosis (1-3). Antiestrogen therapy with tamoxifen, a partial agonist to ER, has had a significant impact on survival in patients with breast cancer, but tamoxifen also has several undesirable side-effects. The antitumor effect of antiestrogen therapy is due to close regulation of breast cell growth by estrogens. However, as breast cancer progresses, it becomes resistant to estrogens, and most patients no longer respond to current antiestrogens (1-3). The continued expression of estrogen and/or progesterone receptors in most patients with tumor progression on tamoxifen indicates that mechanisms for resistance other than receptor loss are common in breast cancer and are responsible for treatment failure (4,5).

This proposal is based on new understanding of the biology of ER, a phosphoprotein that forms a dimer required for binding to specific estrogen response elements in the nucleus, leading to promotion of breast cancer cell growth (6-8). Transcriptional activity of ER is now known to be related to the conformational state of the receptor, especially with respect to the molecular orientation of helix-12 in the ligand-binding domain of ER (8). Helix-12 contains leucine-rich regions that interact with steroid receptor coactivator proteins that, in turn, regulate transcription (9,10) (see Table 1).

TABLE 1. a) Amino acid sequence comparison of AF2 α-helix region in ER from several species, with helix-12 starting at residue 538. Conserved region is in boldface. b) Sequence comparison with ER-ß. c) Sequence alignment of nuclear receptor proteins, including progesterone and androgen receptors (9,11).

a	PL-**YDLLLEML**-DA	535-546	human ER
	PL-**YDLLLEML**-DA	539-550	mouse ER
	PL-**YDLLLEML**-DA	527-538	xenopus ER
b	PV-**YDLLLEML**-NA	433-444	human ER-ß
c	EF-PEMMSEVI-AA	904-915	progesterone receptor
	DF-PEMMAEI I-SV	889-900	androgen receptor

Manipulation of helix-12 interactions with coactivator proteins may provide alternate approaches to anti-hormone therapy. We have synthesized peptides designed to disrupt binding of estrogen receptor with coactivator proteins. Our specific aims for this project included the following experimental objectives:

1) Synthesis of small phosphotyrosyl-peptides targeted to a highly conserved sequence in estrogen receptor including tyrosine-537 and surrounding leucine residues. Experiments evaluated the efficacy of peptide antiestrogens in antagonizing estrogen receptor activity in breast cancer cells, including blockade of estrogen receptor dimerization, reduction of estrogen receptor association with steroid receptor coactivator protein and suppression of estrogen receptor binding to specific estrogen-response elements in DNA.

2) Evaluation of the antitumor efficacy *in vitro* and *in vivo* of small phosphotyrosyl- and malonyltyrosyl-peptides that suppress dimerization and DNA binding of estrogen receptor in human breast cancer cells. Alternative modes for the efficient delivery of low concentrations of peptides *in vivo* will be considered, and effects of peptide antiestrogens on bone, serum cholesterol, uterus and body composition will be evaluated in rodent models.

It is important to develop new antiestrogens which work through different mechanisms of interaction with ER, since these would likely prove useful in treatment of breast cancers that become resistant to conventional anti-estrogens. This project offers an innovative approach to antitumor therapy with the potential for developing novel antiestrogens with minimal toxicity to noncancerous tissues, and it may advance our understanding of the role of estrogen receptor in hormone action (12).

BODY: RESEARCH PROGRESS

AIM 1) Synthesis of small phosphotyrosyl-peptides targeted to tyrosine-537 and the neighboring leucine-rich region in ER and evaluation of their efficacy in the blockade of ER dimerization and ER binding to steroid receptor coactivator and DNA in human breast cancer.

1.a. Peptides disrupt binding of ER with DNA

In order to evaluate potential antiestrogen effects of peptides that mimic the initial sequence in helix-12 in ER, peptides were synthesized by established methods with N-terminal acetylation and a C-terminal amide in the UCLA/Jonsson Cancer Center Peptide Synthesis Facility (12,13). Peptide constructs were characterized by HPLC and mass spectral analysis and found to be > 95% pure. The octapeptide, pY8, contains the sequence:

N-Pro-Leu-*pTyr-Asp-Leu-Leu-Leu-Glu-C (PLpYDLLLE)

and its nonphosphorylated analog, conY8, has the sequence :

N-Pro-Leu-Tyr-Asp-Leu-Leu-Leu-Glu-C (PLYDLLLE).

An additional control peptide with a scrambled sequence, con8, is shown below:

N-Val-Pro-Leu-Asp-Leu-Leu-Leu-Glu-C (VPLDLLLY).

Other peptides of varying size (5-mer and 12-mer) to ascertain the optimal preparation for use in cellular studies have also been prepared (refer to Table 2 in original proposal) (12).

Interaction of ER with nuclear ERE is prerequisite for activation of transcription. To assess specific binding of ER with ERE, we used purified recombinant human ER from MCF-7 breast cancer cells (13). A double-stranded 27-bp probe [5'-GATCCTAGAGGTCACAGTGACCTACGA-3'] encoding the Xenopus vitellogenin A_2 ERE was ^{32}P-end-labeled with polynucleotide kinase. Gel mobility shift assays for the human ER were performed as described (13). The ER in 20 mM reaction buffer (HEPES, pH 7.5, 1 mM EDTA, 100 mM Kcl, 1 mg/ml BSA, 100 nM estradiol, 15% glycerol, and proteinase inhibitors) was incubated with 500 ng of poly (dI-dC) for 15 min at 4 C, and then 20 fmol of the ^{32}P-labeled ERE probe was added for 15 min at 4 C in a total volume of 20 µl. Samples were loaded onto a pre-electrophoresed 5% polyacrylamide gel followed by electrophoresis with cooling at 175 V for 3h in 25 mM TRIS, pH 8.0 with 152 mM glycine and 1 mM EDTA. Under these conditions, the human ER reacts specifically with synthetic ERE in the gel mobility shift assay, allowing formation of an ER-ERE complex (13) (FIG. 1).

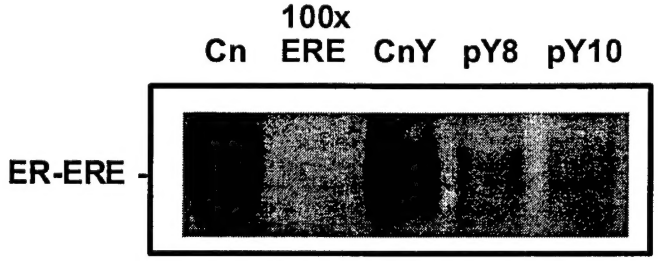

FIG. 1. Gel mobility shift assays of human ER and estrogen-responsive element (ERE). Purified human ER (60 nM) incubated with control solution (Cn), 100-fold molar excess of unlabelled ERE (100x ERE) or with peptides at 2.5 µM. ER was incubated with peptides for 15 min at 4°C, then with 100 nM estradiol-17ß and ^{32}P-ERE. Peptides included peptide pY8, control Y8 peptide (CnY), and a decapeptide, N-Val-Pro-Leu-pTyr -Asp-Leu-Leu-Leu-Glu-Met-C (pY10).

This ER-ERE interaction is blocked by competition with 2.5 µM pY8-peptide but not by competition with 5 µM conY8-peptide (43) or con8-peptide (data not shown). Thus, peptide antiestrogens disrupt ER binding to a specific ERE *in vitro*. However, the IC_{50} for this effect may exceed by 100-fold that required for other cellular actions of the peptides (see ER/SRC-1 interaction below).

1.b. Peptides interfere with ER dimerization

Experiments to assess effects of peptides on ER dimerization, using molecular sizing chromatography with Sephadex G-200 (13,14), were completed. Using this approach, we find that 25 μM pY8 inhibits dimerization of ER (12,13). As with peptide inhibition of ER binding to DNA, peptide interference with ER dimerization appears to occur at a higher IC_{50} than other cellular actions of the peptide (see ER/SRC-1 interaction below). We have confirmed these findings in a previous grant year.

1.c. Peptides block molecular association between ER and steroid receptor coactivator proteins

As noted above, upon activation *in vivo*, ER binds to DNA response elements and recruits co-activator proteins and general transcription factors to form an active complex for stimulation of gene expression. Steroid receptor coactivator-1 (SRC-1) is a well-characterized coactivator protein (165 kd) that mediates steroid hormone responses by promoting receptor-dependent transactivation of genes (15,16), and disruption of the SRC-1 gene results in partial resistance to hormone (16). Short sequence motifs in SRC-1 and other coactivators are necessary to mediate the binding of these proteins to nuclear receptors (10). In order to assess the effect of peptide antiestrogens on the interaction between ER and SRC-1, T47D breast cancer cells were treated *in vitro* with or without 1nM estradiol-17ß, and cell lysates were prepared for immunoprecipitation with antibody to ER, followed by gel electrophoresis and immunoblotting with antibody to SRC-1 as before (17). In the absence of peptide antiestrogens, SRC-1 and ER form a binding complex beginning at 15 min after estrogen treatment, and the association is maximal by 30 min. Prior incubation of breast cells with pY8 interferes with this ER/SRC-1 binding (FIG. 2). In contrast, pre-treatment of T47D breast cancer cells with conY8 or con8 elicits no effect on ER/SRC-1 binding *in vitro* (FIG. 2) (12).

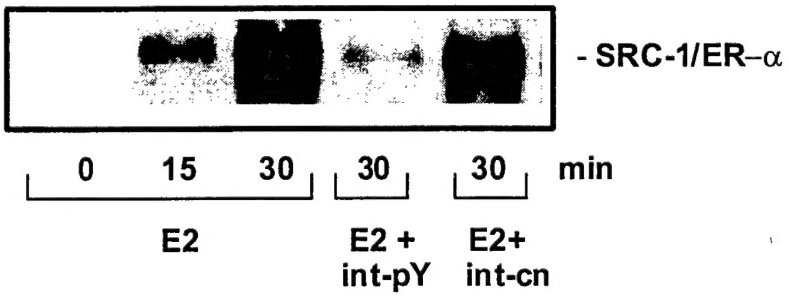

FIG. 2. Effect of estradiol-17ß (E2) on association of ER and SRC-1 in T47D cells. T47D breast cancer cells were treated *in vitro* with 10nM E2 or control vehicle for 15-30 min. For more efficient delivery of peptide, pY8 was coupled with a short peptide from the homeodomain of antennapedia, a vector that promotes internalization of peptides linked to its carboxy-terminus (int-pY8) (18). Int-pY8 or int-peptide control (int-cn) were added 30 min before E2 for an extra 30 min. Lysates were prepared and processed as before. Samples were immunoprecipitated with monoclonal anti-ER antibody-10 (Neomarker), followed by electrophoresis and immunoblot with monoclonal anti-SRC-1 antibody (Affinity Bioreagents).

Thus, tyrosine-537 and adjacent leucine residues in ER may be important in regulating ER/SRC-1 interaction in breast cancer. In contrast to peptide effects on ER dimerization and DNA binding *in vitro*, the peptide effect on ER/SRC-1 association can be elicited at nanomolar concentrations of active peptides. Thus, peptide blockade of the binding of receptor coactivator protein with ER may have more physiologic significance *in vivo*.

AIM 2) Evaluation of the antitumor efficacy in vitro and in vivo of phosphotyrosyl- and malonyltyrosyl-peptides that suppress biologic activity of ER in human breast cancers

2.a. Peptide antitumor effects *in vitro* and *in vivo*

One problem with use of phosphotyrosyl-peptides *in vitro* or *in vivo* is susceptibility of the constructs to degradation by cellular tyrosine phosphatase enzymes. To address this difficulty, we prepared phosphotyrosyl-mimic peptides that use malonic acid rather than phosphate residues at tyrosine sites. Malonyl-tyrosine residues appear to mimic the phosphotyrosine conformation in proteins and evade the action of cellular enzymes targeted to phosphotyrosine (19,20). The malonyltyrosyl-peptides contain the sequence surrounding tyrosine-537 in ER (12,13) (see Table 1). Malonyltyrosyl-octapeptide, mY8, was synthesized by established methods and contains the sequence:

N-Pro-Leu-mTyr-Asp-Leu-Leu-Leu-Glu-C (PLmYDLLLE).

We find that malonyltyrosyl-peptide constructs, as phosphotyrosyl-peptides, suppress binding of ER to specific ERE in human breast cancer cells. This ER-ERE interaction is blocked by competition with 2.5 μM mY8-peptide but not by 5 μM conY8-peptide (12,13). Our studies indicate that 8-12-mer malonyltyrosyl-peptides are the optimal peptide sequence for use in *in vivo* studies.

Using the pY8-internalization vector (int-pY8), we find that nanomolar concentrations of the peptide have good efficacy in disruption of estrogen-induced growth of human breast cancer cells (12). The anticipated growth stimulation by estrogen is found after treatment of MCF-7 cells with control internalization peptide alone, exceeding growth of control cells in the absence of estrogen by 3-fold. Similarly, a low concentration of free pY8-peptide alone in solution (25 μM) does not alter the growth response to estrogen. However, peptide antiestrogen coupled with internalization peptide suppresses the expected growth effect of estrogen ($P<0.001$). A dose-response study using concentrations of drug ranging from 0.02 to 500 nM shows that the pY8-internalization peptide is effective in growth inhibition of MCF-7 cells at concentrations < 25 nM (12). Studies of breast tumor xenografts *in vivo* have been done using methods as before (17) with peptide delivery by IP injection (13,17). However, in these experiments, we have encountered considerable difficulty in obtaining uniform antitumor efficacy, a result that may be due to biologic variabilities in peptide bioavailability and administration.

2.b. Peptidomimetic derivatives to disrupt biologic activity of estrogen receptor

Delivery of peptides in the clinic may be problemsome (24). Therefore, we have assessed the potential use of more lipophilic derivatives that mimic the activity of peptide antiestrogens but may be easier to administer. In collaborative studies with Dr. Michael Jung in the Department of Chemistry at UCLA, we have synthesized small steroidal and non-steroidal compounds targeted to disrupt the biologic activity of the helix 12 region in ER. The choice of our target molecules is based on structural information given in x-ray structures of the ligand-binding domain of the estrogen receptor with both estradiol and raloxifene. One requires two OH groups (hydroxyl or phenol) placed at the right distance in order to bind to the receptor but, in addition, to cause the helix 12 to assume a different conformation, one requires also an additional binding element, namely a correctly disposed tertiary amine, to bind to Asp 351. We have prepared several steroidal and non-steroidal compounds all of which have both the required diol unit properly spaced along with a variable length alkyl chain containing a terminal tertiary amine (or guanidine unit) to bind to Asp 351 and thereby induce the conformation change in order to shift helix 12.

Candidate antiestrogens prepared in the Chemistry Laboratory were evaluated for estrogen binding activity by use of established [^{3}H]-estradiol-17ß competition binding assays. Results of experiments showing specific binding of [^{3}H]-estradiol-17ß with human MCF-7 breast cancer cells are shown in FIG. 3.

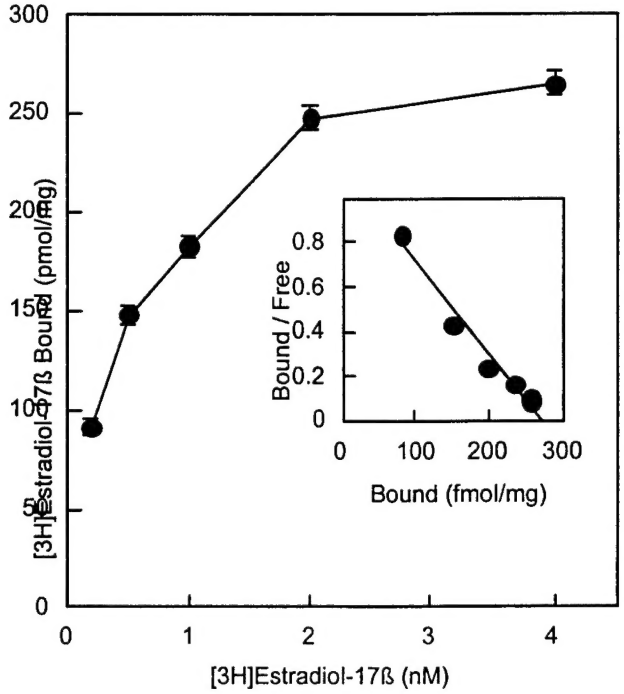

FIG. 3. Binding of [^{3}H]estradiol-17ß by MCF-7 human breast cancer cells. Specific binding of [^{3}H]estradiol-17ß by MCF-7 cells was determined by methods described elsewhere (25). A 100-fold molar excess of unlabeled estradiol-17ß was present with [^{3}H]estradiol-17ß in paired samples for determination of displaceable binding. Specific binding of estradiol by intact cells is shown. Scatchard analyses of the binding data to determine estrogen-binding capacity (B_{max}) and the affinity of hormone binding (K_d) are shown in the inset. The K_d of estradiol binding to MCF-7 cells was 2.5 x 10^{-10} M, and the estradiol binding capacity in MCF-7 cells was 270 fmol/mg protein. These values are based on results from three experiments.

Data from screening experiments to assess competition for [^{3}H]-estradiol-17ß binding are presented in FIG. 4. Of fifteen additional compounds tested, several showed evidence of significant competition for specific [^{3}H]-estradiol-17ß binding with intact MCF-7 cells *in vitro*. Experiments to measure competition of the test compounds for specific[^{3}H]-estradiol-17ß binding with particle-free cytosol fractions from MCF-7 cells are consistent with these findings.

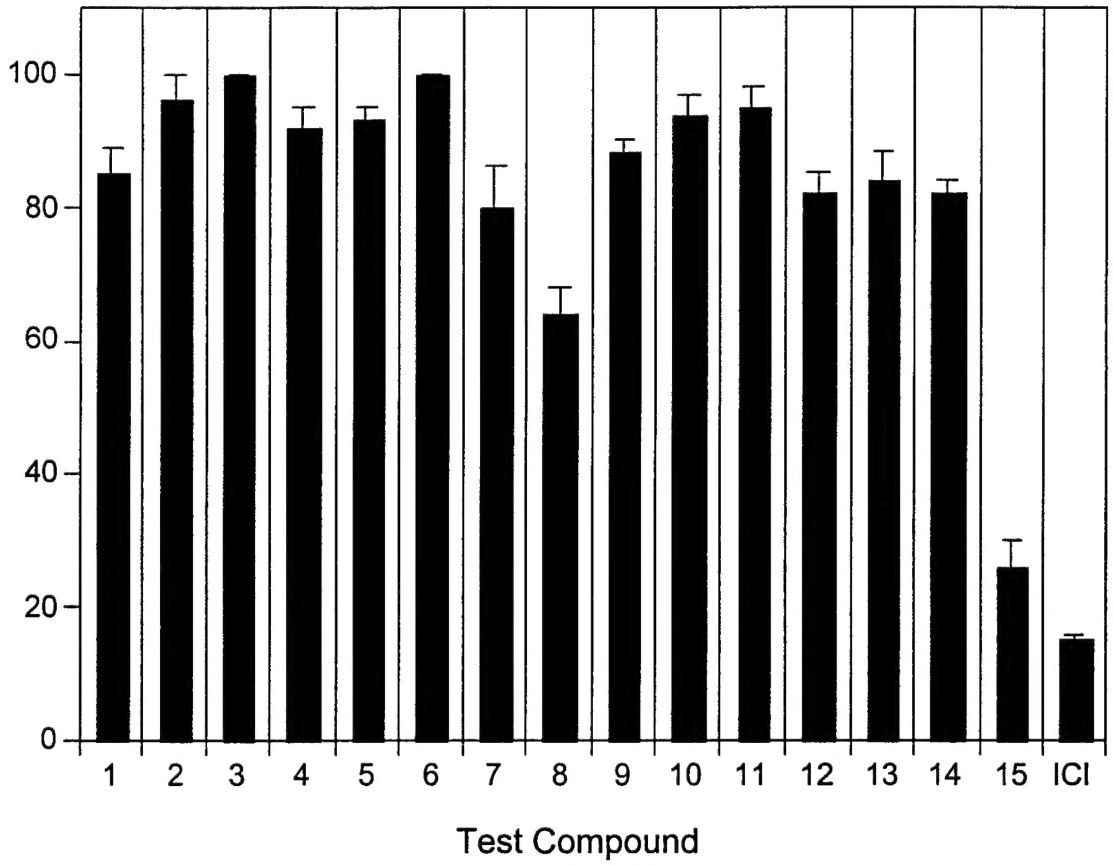

FIG. 4 Inhibitory effects of new peptidomimetic test compounds on specific [3H]-estradiol-17ß binding with MCF-7 breast cancer cells *in vitro*. Specific binding of 2 nM [3H]-estradiol-17ß was assessed. To determine competition for binding, test compounds included steroid derivatives 1-15 or ICI 182,780 (Faslodex), a known steroidal antiestrogen, at a concentration of 1 μM.

Compounds with significant biologic activity in these screening assays were selected for further study of antitumor activity using MCF-7 human breast cancer cells *in vitro*. The results of studies on proliferation of MCF-7 cells are shown in FIG. 5. Two compounds significantly block growth of the breast cancer cells in this *in vitro* assay, and the extent of growth inhibition exceeds that found with Faslodex and tamoxifen at equivalent doses. We have further assessed antiestrogen S15 for biologic efficacy using assay methods outlined in the original Preliminary Results section. Antiestrogen S15 elicits relatively weak suppression of estrogen receptor-induced transcriptional activity using an ERE-CAT reporter gene approach (FIG. 6). However, antiestrogen S15 is a strong inhibitor of estrogen-induced stimulation of the phosphorylation of MAP kinase, a membrane-initiated signaling pathway that correlates well with growth regulation (25) (FIG. 7). One further test of antiestrogenic activity will be assessment of the molecular association between ER and the steroid receptor coactivator protein, SRC-1, as described previously. Compounds with significant biologic activity in screening assays, such as S15, will be selected for studies of antitumor activity using MCF-7 human breast cancer cells *in vivo*. These results will be compared with those from experiments with corresponding peptide antiestrogens.

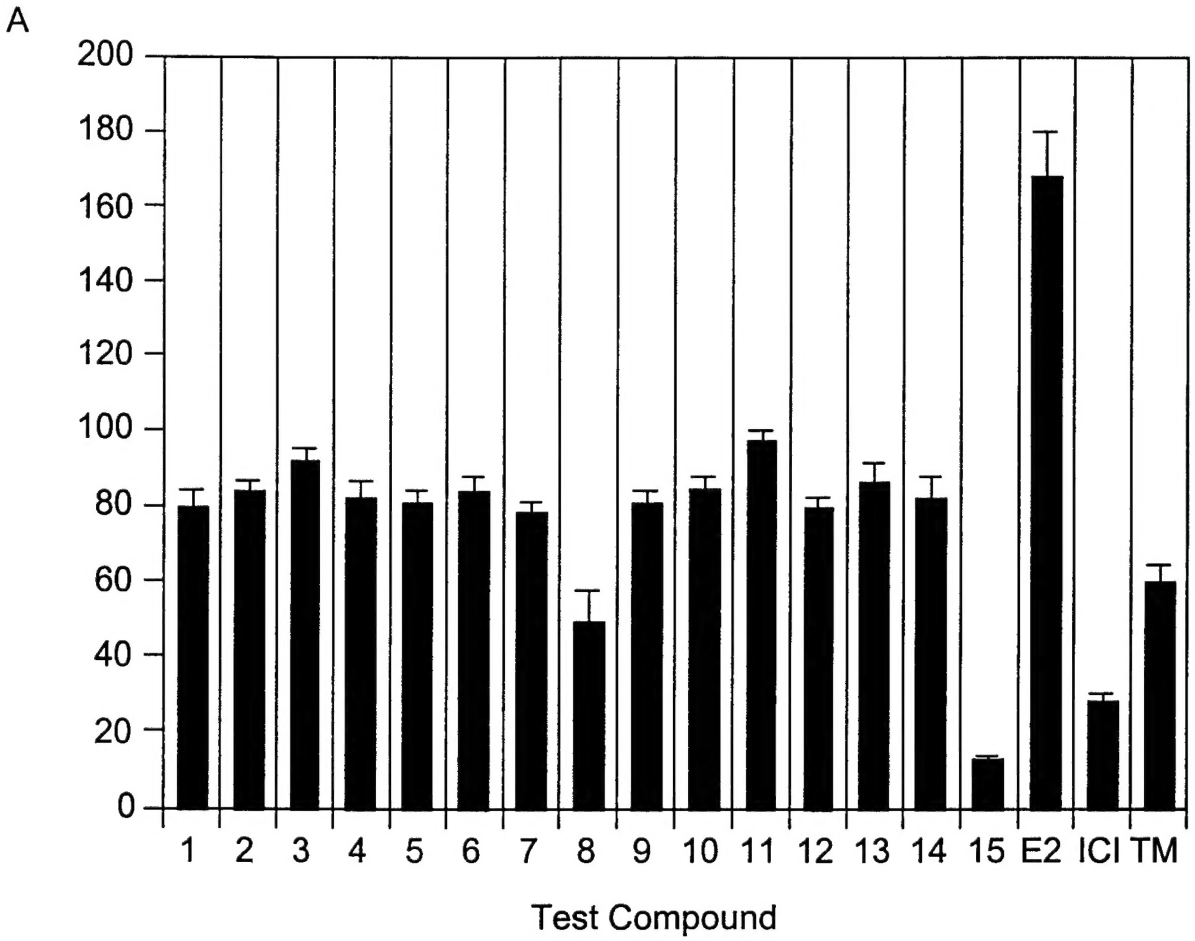

FIG. 5 Effects of new peptidomimetic test compounds 1-15, estradiol-17ß, ICI 182,780 and tamoxifen on growth of MCF-7 cells *in vitro*. Growth assays were done as before (*25*). Test compounds were administered at 1 μM, with estradiol-17ß (E2) at 10 nM, ICI 182,780 (ICI; Faslodex) at 10 nM and tamoxifen at 1 μM. The results are from triplicate experimental determinations.

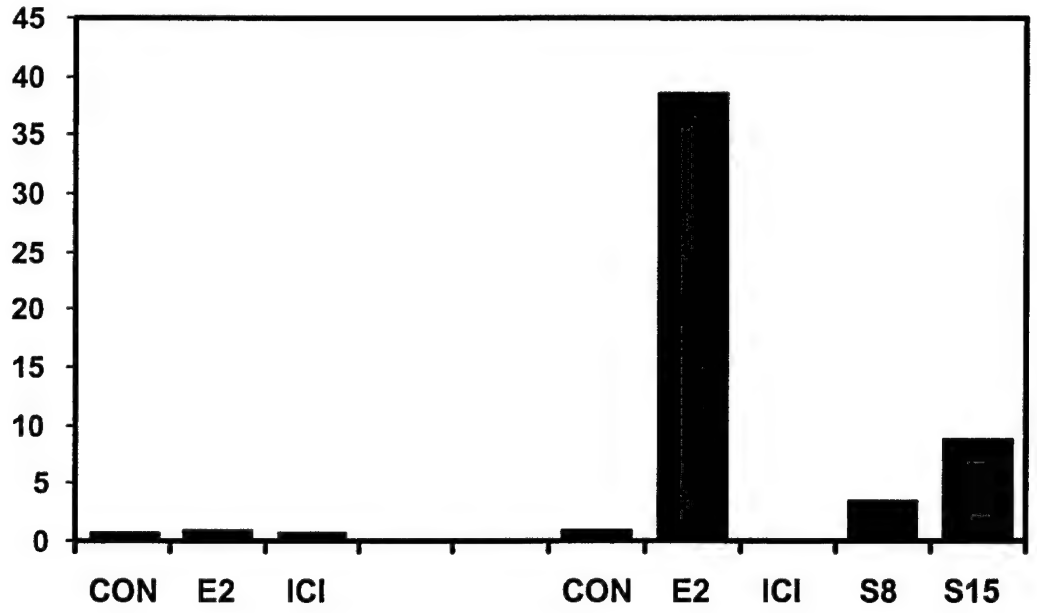

FIG. 6 Effects of peptidomimetic antiestrogens (S15, S8) on estrogen-induced ERE-CAT reporter gene activity in COS-7 cells without or with transfected ER (17, 25). Antiestrogen S15 elicits a relatively mild effect as compared with the activity of the pure antiestrogen, ICI 182,780 (Faslodex).

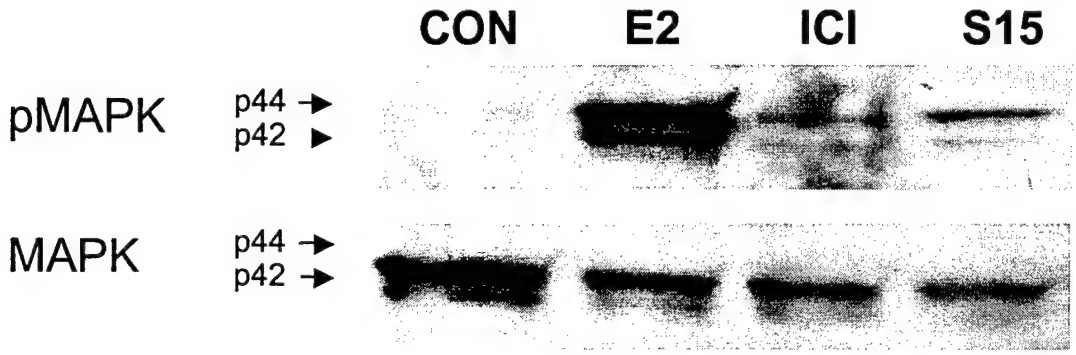

FIG. 7 Effects of peptidomimetic antiestrogens on estrogen-induced phosphorylation of MAP kinase in MCF-7 breast cancer cells, using established methods (17,25). Antiestrogen S15 is effective in the suppression of the estrogen-induced activation of MAP kinase (25).

2.c. Tissue selectivity of synthetic antiestrogens

Estrogen is a key regulatory hormone which, in addition to its role in reproductive tissues, affects a number of physiological systems, including the skeleton and cardiovascular system. In order to evaluate tissue selectivity of the final peptide and peptidomimetic steroidal formulations, these agents will be given to immature female mice for 3 days, and effects on uterine wet and dry weight will be assessed (23). In addition, uterine hypertrophy will be evaluated in older (up to 15-mo-old) female mice treated with peptides for 28-90 days. In preliminary experiments, no uterine hypertrophy was found on treatment with either peptide or non-steroidal antiestrogen compounds. Effects of peptides on total serum cholesterol, fat body mass and lean body

mass in these aged female rodents are being completed. In future studies with ovariectomized female mice, we plan to assess effects of peptides on ovariectomy-induced increments in body weight gain, serum cholesterol, and bone loss. Following ovariectomy, rodents will be assigned to control groups including both placebo and positive control with estrogen replacement using established methods (23, 25). Our goal is to develop novel antitumor agents with minimal toxicity to noncancerous tissues, and these experiments will help to establish the response profile and tissue selectivity of peptide as well as non-steroidal antiestrogens.

KEY RESEARCH ACCOMPLISHMENTS

- Small leucine-rich peptides that mimic ER sequence at the start of helix-12 reduce the formation of ER homodimers and reduce binding of ER to ERE.
- Small leucine-rich peptides that mimic ER sequence at the start of helix-12 suppress association of ER with SRC-1.
- Estrogen-dependent growth of human breast cancer cells is blocked by pre-treatment with small leucine-rich peptides that mimic ER sequence at the start of helix-12.
- Small non-steroidal, peptidomimetic compounds have strong antitumor activity in estrogen-dependent breast cancers and are easier to administer than peptide compounds.

REPORTABLE OUTCOMES

Presentations

1. "Small Molecule Inhibitors of Estrogen Receptor Function". Presented at Molecular Oncology Seminar, Genentech, South San Francisco (1999).

2. "Peptide antagonists of the estrogen receptor block growth of human breast cancer cells". Presented at Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta (2000).

3. "Interactions between Type I receptor tyrosine kinases and steroid hormone receptors : Therapeutic implications". Presented at First International Symposium on Translational Research in Oncology, Dublin, Ireland (2001).

4. "Peptide antiestrogens block growth of human breast cancer cells". Presented at Jonsson Cancer Center Seminar Series in Oncology, UCLA (2002).

Abstracts

1. Pietras, R.J., Marquez, D., Chen, X. and Li, D. (2000). Peptide antagonists of the estrogen receptor block growth of human breast cancer cells. Era of Hope DOD Breast Cancer Research Program Proceedings, **2**: 535.
2. Marquez, D.C., Chen, H.-W., Tinder, R., Sugiyama, S., Jung, M.E. and Pietras, R.J. (2002). Development of function-specific antiestrogens to block signal transduction by membrane-associated estrogen receptors in human breast cancer cells. Proceedings Endocrine Society Annual Meeting.

Publications

1. Pietras, R.J., Nemere, I. and Szego, C.M. (2001). Steroid hormone receptors in target cell membranes. Endocrine **14** : 417-427.
2. Marquez, D.C., Lee, J., Lin, T. and Pietras, R.J. (2001). Epidermal growth factor receptor and tyrosine phosphorylation of estrogen receptor. Endocrine **16** : 73-81.
3. Szego, C.M., Pietras, R. and Nemere, I. (2002). Plasma membrane receptors for steroid hormones: Initiation site of the cellular response. Encyclopedia of Hormones (in press).

Patents

A United States patent, number 6,306,832, and entitled, "Peptide antiestrogen compositions and methods for treating breast cancer", was granted for work related to these studies on October 23, 2001.

No degrees, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

This project is a new approach to antitumor therapy with the potential for developing antiestrogen treatments with minimal toxicity to noncancerous tissues. Small leucine-rich peptides that mimic ER sequence at the start of helix-12 in the receptor molecule are especially effective in suppressing the association of ER with SRC-1. This molecular action appears to elicit blockade of breast cancer cell proliferation. The results of our studies suggest that treatment with small peptide antiestrogens may prove more effective than drugs currently available in blocking the growth-promoting signals of estrogen receptors. This work provides good evidence of target validation for helix-12 in estrogen receptor. Since bioavailability and administration of these peptides may be problemsome in the clinic, we have prepared non-steroidal, peptidomimetic compounds that have comparable antitumor efficacy in estrogen-dependent breast cancers and are easier to administer.

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PERSONNEL

During the course of the complete grant period, the following personnel were engaged in this project:

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Presented at Endocrine Society Annual Meeting, 2002 :

Development of Function-Specific Antiestrogens to Block Signal Transduction by Membrane-Associated Estrogen Receptors in Human Breast Cancer Cells. Diana C Marquez[1], Hsiao-Wang Chen[1], Robert Tinder[2], Shigeo Sugiyama[2], Michael E Jung[2], Richard J Pietras[1]. [1]*Department of Medicine-Hematology/Oncology and* [2]*Department of Chemistry and Biochemistry, UCLA, Los Angeles, CA.*

Specific membrane binding-sites for estrogen have been described in MCF-7 breast cancer cells (Oncogene 20: 5420, 2001). These membrane proteins with high affinity and specificity for binding estradiol-17ß (E2) trigger rapid signaling cascades after E2 stimulation that promote cell growth. The aim of this study is to develop new antiestrogens capable of interacting with different signaling pathways mediated by either nuclear or membrane forms of estrogen receptors (ER). A number of steroidal antiestrogens were designed and tested *in vitro* using assays with specificity for membrane-associated or nuclear receptor signaling. One such derivative with structural modification of estradiol-17ß (E2) at position C-11 was designated S15. MCF-7 human breast cancer cells were grown in estrogen-depleted media prior to binding assays. S15 displaced total cell [^{3}H]-E2 binding in a dose-dependent manner by 22% at 1 μM, 50 % at 5 μM and 80% at 10 μM concentration (all at $p<0.05$). Stimulation of MCF-7 cell proliferation induced by 10 nM E2 was suppressed when cells where incubated in the presence of 10 μM S15 ($p= 0.01$). To determine if the effect of S15 was mediated by enhanced transcription via a classical estrogen response element (ERE), COS-7 cells without ER were co-transfected with ER expression vector and an ERE-CAT reporter gene. After transfection, COS-7 cells were incubated with 1nM E2 and 10 μM S15. Under these conditions, S15 elicited only a partial reduction in ERE-CAT transcriptional activity as compared to the response with E2 alone. However, S15 elicited a more profound inhibition of membrane-associated signaling. To assess membrane-associated signal transduction, MCF-7 cells were incubated in the presence of 10 μM S15 and 1 nM E2. Cells were then homogenized, with lysates subjected to Western Blot analysis. Using an anti-phospho-p44/42 MAP kinase antibody, enhanced phosphorylation of MAPK was found 2 min after E2, but this stimulatory effect of E2 was blocked by S15. As determined from results of subcellular fractionation studies with MCF-7 cells, these primary agonist-antagonist effects may be initiated in lipid raft signaling platforms at the surface membrane. Further development of new therapeutics that differentiate between nuclear and membrane-associated forms of ER may be important for breast cancer treatment. [Grants from Susan G. Komen Breast Cancer Research Foundation, US Army BCRP and Stiles Program in Integrative Oncology].

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Epidermal Growth Factor Receptor and Tyrosine Phosphorylation of Estrogen Receptor

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Activation of estrogen receptor-α (ERα) by growth factors in the absence of estrogen is a well-documented phenomenon. To study further this process of ligand-independent receptor activation, COS-7 cells without ER were transfected with both ER and epidermal growth factor receptor (EGFR). In the absence of estrogen, epidermal growth factor (EGF) stimulated rapid tyrosine phosphorylation of ER in transfected COS-7 cells. Similarly, in MCF-7 breast cancer cells that have natural expression of ER and EGFR, EGF promoted acute phosphorylation of serine and tyrosine residues in ER, and a direct interaction between ER and EGFR after treatment with EGF was found. In confirmation of a direct interaction between ER and EGFR, activation of affinity-purified EGFR tyrosine kinase in vitro stimulated the phosphorylation of recombinant ER. The cross-communication between EGFR and ER appears to promote significant stimulation of cell proliferation and a reduction in the apoptotic loss of those cells that express both receptor signaling pathways. However, COS-7 cells transfected with both ER and EGFR show minimal stimulation of classical estrogen response element (ERE)-dependent transcriptional activity after stimulation by EGF ligand. This suggests that the proliferative and antiapoptotic activity of EGF-induced ER activation may be dissociated from ERE-dependent transcriptional activity of the ER.

Key Words: Epidermal growth factor; estrogen receptor; tyrosine phosphorylation; estradiol; MCF-7 cells; apoptosis.

Introduction

The estrogen receptor (ER) is a member of a large family of nuclear receptors that share a common structural and functional organization. These receptors are generally considered to function as ligand-activated transcription factors *(1–3)*. However, accumulating evidence has demonstrated significant cross-communication between steroid hormone receptors and peptide growth factor signaling pathways, with some reports suggesting that growth factors may promote activation of steroid receptors even in the absence of natural ligand. Agents capable of exerting such ligand-independent activation of ER include epidermal growth factor (EGF) *(4–9)*, transforming growth factor-α *(7)*, heregulin *(10)*, insulin *(11)*, insulin-like growth factor-1 *(7,8,12–14)*, and dopamine *(15)*. Under estrogen-free conditions, *in vivo* administration of EGF alone mimics the effects of estrogen in the mouse reproductive tract *(16,17)*. In mice lacking ER-α expression, both estrogen- and EGF-stimulated uterine growth is blocked *(17)*. Thus, ER may mediate the transcription of target genes by integrating signals from growth factor–activated pathways as well as from steroid hormone binding *(18)*.

It is notable that cooperative interactions between erb B and nuclear receptors were first reported more than a decade ago *(19)*. The EGF receptor (EGFR) is a 170-kDa transmembrane glycoprotein that consists of an extracellular ligand-binding domain in its amino terminus, a transmembrane-spanning region, and a cytoplasmic EGF-stimulated protein tyrosine kinase in its C-terminus. EGFR is part of the erb B family of growth factor receptors. On ligand binding and dimerization, the receptor undergoes phosphorylation on tyrosine residues. EGFR activation results, in turn, in the phosphorylation of downstream protein kinases and the subsequent activation of specific transcription factors. With emerging evidence for estrogen-stimulated activation of mitogen-activated protein kinase (MAPK) signaling pathways *(8)*, growth factor- and steroid hormone–dependent mitogenic cascades may well have significant interactions.

The ER is characterized by six major functional domains often termed A–F. The A/B region contains an N-terminal transactivation domain, AF-1; the C region harbors the DNA-binding domain, while the D-region is involved in nuclear localization signaling; and E/F contains the C-terminal portion of the receptor and is involved in hormone binding, dimerization, and the function of a second transactivation domain, AF-2 *(2,3,20)*. AF-1 and AF-2 appear to contribute synergistically to the transcription of ER-regulated target genes, but they have different mechanisms of activation. AF-1 activity is highly dependent on serine phosphorylation by MAPK signaling *(8)*, while AF-2 is activated

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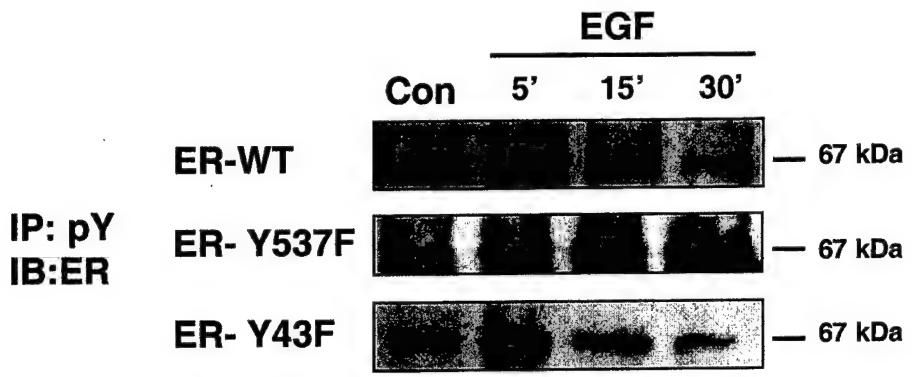

Fig. 1. EGF treatment promotes tyrosine phosphorylation of ER. COS-7 cells were transfected with EGFR and ER vectors and then treated with control vehicle (Con) or 2 n*M* EGF for 5, 15, and 30 min. Cell lysates were processed as described in Materials and Methods, then immunoprecipitated (IP) using an antiphosphotyrosine antibody (pY), before electrophoresis and immunobloting (IB) with anti-ER antibody (ER). Treatment groups included COS-7 cells transfected with EGFR + ER-wild type (WT), EGFR + ER-Y537F mutant (Y537F), and EGFR + ER-Y43F mutant (Y43F). A representative blot from one of three experiments is shown.

by binding estrogenic ligands. EGF-stimulated activation of ER may be mediated, in part, by the AF-1 domain of ER. Within the AF-1 domain, phosphorylation of serine-118 appears to be required for full activity of AF-1, and this phosphorylation step is mediated by MAPK *(8,9,21)*. Additional phosphorylation sites in ER that may participate in the transcriptional activation of ER include serine-167, a major estradiol-induced phosphorylation site on ER *(22)*, as well as serine-104 and serine-106 *(23)*.

Several reports have also provided evidence for significant phosphorylation of the ER at tyrosine residues *(10, 24–26)*. Although a number of initial studies suggested that phosphorylation of ER at tyrosine-537 (Y537) may be important for DNA binding and for transcriptional activation *(25–28)*, more recent evidence indicates that phosphorylation at Y537 of ER is not an absolute requirement for hormone binding to ER or for activation of ER-dependent transcription *(29,30)*. However, the role of ER tyrosine phosphorylation sites in the regulation of cell proliferation and in the cellular response to growth factor stimulation *(24,31)* has not been fully evaluated.

To assess the hypothesis that EGF-mediated activation of ER may involve tyrosine phosphorylation of ER, we used several different experimental approaches to evaluate cross-communication between ER and EGFR. The combined results suggest that EGFR tyrosine kinase interacts directly with ER in solution and in intact cells, leading to tyrosine phosphorylation of ER. This alteration in ER may then contribute to the promotion of estrogen-independent activation of ER-mediated transcription and cell proliferation.

Results

EGF Treatment Promotes EGFR-Mediated Tyrosine Phosphorylation of ER in Intact Cells

Previous work has demonstrated that ER can undergo tyrosine phosphorylation in a process that appears to be mediated by cellular tyrosine kinase receptors *(10,24–26, 30)*. To determine whether tyrosine phosphorylation of ER can be mediated by EGFR, COS-7 monkey kidney cells with low to nil EGFR and no ER were transiently transfected with expression vectors for EGFR and ER-wild type and then treated, in the absence of estrogen, with 2 n*M* EGF. The results showed that ER-wild type is tyrosine phosphorylated after cell stimulation with EGF in the absence of estrogen (Fig. 1). The level of ER phosphorylation increased significantly by 5 min and then declined after 30 min. To assess the contribution of tyrosine-537 in ER in this process, COS-7 cells were next transfected with EGFR and ER with directed mutation of tyrosine-537 to phenylalanine (Y537F). The mutated ER-Y537F showed a modest increase in basal levels of ER phosphorylation (Fig. 1). In addition, cells transfected with ER-Y537F exhibited a reduction in the level of receptor phosphorylation at 5 min after EGF treatment but no apparent decrease at later times (Fig. 1). This result suggests that this is not the tyrosine residue that is primarily phosphorylated in ER or that more than one tyrosine residue in ER may be phosphorylated *(30)*. To evaluate the potential role of other tyrosine residues in ER, COS-7 cells were transfected with EGFR and ER with a directed mutation of tyrosine-43 to phenylalanine (Y43F). This alteration elicited an increase in the basal level of tyrosine phosphorylation of ER. In addition, the EGF response of COS-7 cells containing EGFR and ER-Y43F appeared more deficient, especially when compared with control (Fig. 1). These findings may indicate that tyrosine residues other than the 537-residue may participate in EGFR-mediated phosphorylation of ER.

EGF Stimulates Low Levels of Estrogen Response Element–Dependent Transactivational Activity of ER in Absence of Estrogen

The effects of EGF and estrogen on transcriptional activation of an estrogen response element (ERE) were assessed using a reporter plasmid, pERE-BLCAT, containing the vitellogenin A2 ERE *(32)*. COS-7 cells were transfected with

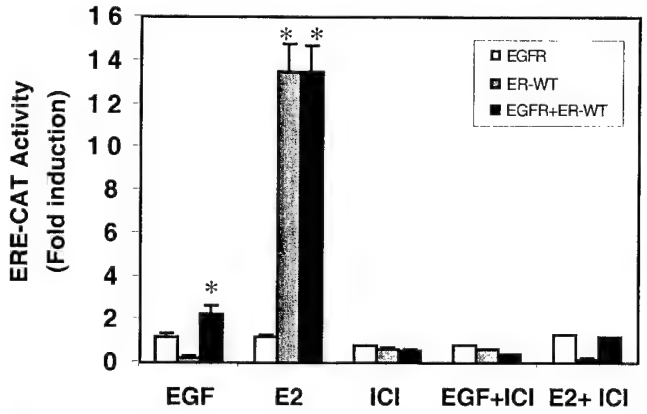

Fig. 2. EGF stimulates a low level of transactivation activity of ER in the absence of estrogen ligand. COS-7 cells were transfected with EGFR and pERE-BLCAT reporter gene (EGFR); ER and pERE-BLCAT (ER-WT); or EGFR, ER, and pERE-BLCAT (EGFR + ER-WT). Cells were treated with control vehicle, 2 n*M* EGF, 10 n*M* estradiol 17-β (E_2), 1 μ*M* ICI 182,780 (ICI), or combinations of these reagents for 18 h. After treatment, cell lysates were prepared and analyzed for ERE-CAT activity by established methods. Transactivation of the CAT reporter gene is expressed as fold induction of the untreated control. Each bar represents the mean ± SE of determinations from three individual experiments. Asterisks denote results significantly different from control at $p < 0.05$.

ERE-chloramphenicol acetyltransferase (CAT) reporter gene in combination with either EGFR alone or EGFR plus ER-wild type (ER-wt). Treatment with estradiol-17β induced transactivation of the ERE-CAT reporter in cells transfected with ER-wt by about 14-fold ($p < 0.001$) (Fig. 2). By contrast, treatment with EGF elicited ER transactivation by only about two-fold ($p < 0.05$) in cells transfected with ER-wt and not at all in those cells transfected with EGFR alone. Of importance, ER transactivation induced by estradiol and by EGF were both inhibited by coadministration of the pure antiestrogen ICI 182,780 *(33)*, thus suggesting that these activities are mediated by ER.

EGF Treatment Promotes Interactions Between EGFR and ER in Human Breast Cancer Cells

To assess the potential direct interaction between EGFR tyrosine kinase and naturally expressed ER in intact cells, MCF-7 human breast cancer cells known to express significant levels of EGFR *(34)* were treated with 2 n*M* EGF for 1–60 min in vitro. Thereafter, the cells were disrupted and processed for immunoprecipitation with anti-EGFR antibodies and then immunoblotting with anti-ER antibodies (Fig. 3A). The results showed an enhanced interaction between ER and EGFR that was evident by 1 min after EGF treatment, followed by a peak at 15–30 min and then a decline to baseline levels of receptor association by 60 min (Fig. 3A). As an additional control, the treated membrane was stripped and reprobed using anti-EGFR antibody to confirm that EGFR did not significantly vary during the course of the experiment (Fig. 3B). The time course of the direct interaction between ER and EGFR was compared with the known phosphorylation of serine residues in ER (Fig. 3C) and the phosphorylation of tyrosine residues in ER (Fig. 3C) after treatment of MCF-7 cells with 2 n*M* EGF in vitro.

EGF Stimulation of EGFR Promotes Phosphorylation of ER in Solution

To assess further the interaction of EGFR tyrosine kinase with ER, these proteins were studied in solution in vitro. It is notable that EGF stimulation of immunoaffinity-purified EGFR kinase activity induces a significant increase in EGFR autophosphorylation *(35–37)*, a phenomenon observed in the present experiment (Fig. 4). Incubation of the affinity-purified human EGFR with purified recombinant human ER in the presence of estrogen and EGF induced significant phosphorylation of ER in the absence of any other cellular kinase enzymes in solution (Fig. 4). The level of ER phosphorylation was substantially higher than that found in the absence of EGFR. The added phosphorylation is likely owing to derivatization of tyrosine residues in ER by the action of EGFR tyrosine kinase.

EGF-Induced Cell Proliferation is Enhanced and Cell Death is Reduced When Both EGFR and ER Are Present

EGF *(38–40)* and estrogen *(41)* are both known mitogens for breast cancer cells. To assess the potential contribution of EGFR signaling pathways in ER-mediated cell growth, COS-7 cells were transiently transfected with either control vectors, EGFR vector alone, ER-wt vector alone, or both receptor vectors. Under these conditions, treatment with EGF elicited no significant stimulation of the growth of parental or mock-transfected COS-7 cells, nor COS-7 cells transfected only with ER-wt ($p > 0.05$) (Fig. 5A). By contrast, EGF markedly enhanced the growth of EGFR-transfected COS-7 cells to about 1.6 times that of controls ($p < 0.05$) (Fig. 5A). Cell proliferation induced by EGF was further enhanced to about 2.1 times that of controls when both ER-wt receptors and EGFR were cotransfected in COS-7 cells ($p < 0.01$) (Fig. 5A). A modest reduction in

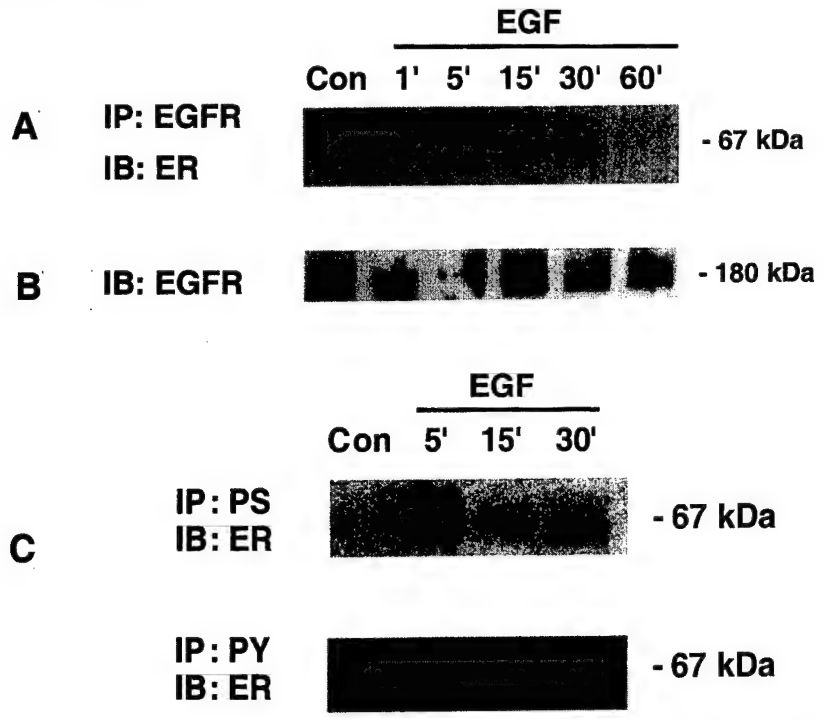

Fig. 3. EGF treatment of MCF-7 human breast cancer cells promotes association of EGFR with ER and stimulation of ER phosphorylation. (**A**) EGF treatment promotes association of EGFR with ER in MCF-7 cells. MCF-7 cells were treated with control vehicle (Con) or 2 n*M* EGF for 1, 5, 15, and 30 min. Cell lysates were prepared and processed as described in Materials and Methods. Immunoprecipitation (IP) was done using anti-EGFR antibody before electrophoresis, and immunobloting (IB) was done with anti-ER antibody. A representative blot from one of six experiments is shown here. (**B**) EGFR in MCF-7 cells. As an additional control experiment, treated membrane from panel (**A**) was stripped and reprobed with anti-EGFR antibody to ensure no significant variation in EGFR during the course of the treatment. (**C**) EGF treatment promotes phosphorylation of serine and tyrosine residues in ER. MCF-7 cells were treated with control vehicle (Con) or 2 n*M* EGF for 5, 15, and 30 min. Cell lysates were prepared and processed as described in Materials and Methods. IP was done using either antiphosphoserine (PS) or antiphosphotyrosine (PY) antibody before electrophoresis, and IB was done with anti-ER antibody.

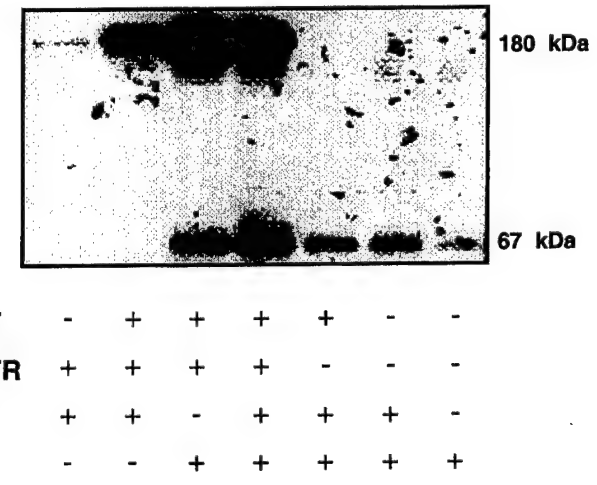

Fig. 4. Phosphorylation of purified recombinant ER in vitro by activated affinity-purified EGFR tyrosine kinase. ER, EGFR, or both receptor proteins in the presence of 100 n*M* estradiol 17-β (E_2), 100 n*M* EGF, or both ligands in solution were incubated in vitro. After the addition of 10 μ*M* ATP and 1 μCi (6000 Ci/mmol) of [δ-^{32}P]-ATP, samples were incubated at 5°C for 15 min. Proteins were separated on 7.5% SDS-PAGE gels, and after running, gels were dried and exposed for autoradiographic analysis using established methods. A representative film from three experiments is shown.

the anticipated level of EGF-stimulated cell growth occurred when COS-7 cells were transfected with EGFR in combination with ER isoforms mutated at tyrosine-537 ($p > 0.05$) (see Fig. 5A). Moreover, COS-7 cells transfected with EGFR and ER forms mutated at tyrosine-537 showed significantly less proliferation in response to EGF stimulation than those cells containing a combination of ER-wt receptors and EGFRs ($p < 0.05$).

Since cumulative cell growth is a function of both cell proliferation and cell loss *(42–44)*, EGF-induced inhibition of cell death was also assessed using a modified TdT-mediated dUTP nick-end labeling (TUNEL) assay *(45)* in COS-7 cells grown in vitro under growth factor–depleted conditions (Fig. 5B). The cells were first plated in standard media for 48 h, and then the media were changed to phenol-red free media containing 0.1% dextran-coated, charcoal-treated fetal bovine serum (DCC-FBS) to promote estrogen-free and serum-depleted conditions. EGF-induced blockade of apoptosis was assessed in COS-7 cells in the native state or transfected with control vector, EGFR, EGFR and ER-wt, EGFR and ER-Y537F mutant, or EGFR and ER-Y537A mutant. After transfection, cells were treated with 10 n*M* EGF and cultivated 72 h before TUNEL assay,

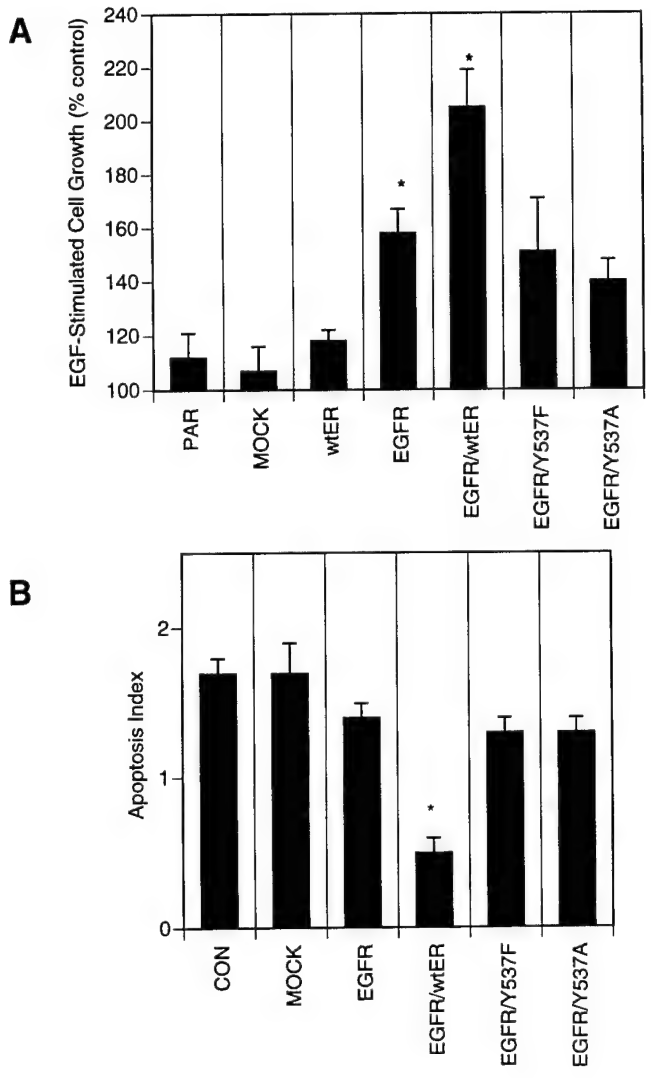

Fig. 5. EGF treatment stimulates enhanced proliferation and reduced apoptosis of COS-7 cells transfected with EGFR and ER. (**A**) EGF-induced cell growth was assessed in COS-7 cells in the native state (PAR) or transfected with control vector (MOCK), ER-wild type (wtER), ER-Y537F mutant (Y537F), ER-Y537A mutant (Y537A), EGFR, or combinations of the receptor vectors. After transfection, cells were treated with control vehicle alone or 10 n*M* EGF. Cells were then cultivated further, and final cell numbers were quantitated after 72 h for each treatment group as indicated. Data (mean ± SE) were collected from 10 to 20 independent experiments. (**B**) EGF-induced inhibition of cell death was assessed using a modified TUNEL assay *(45)* in COS-7 cells. The cells were first plated in standard medium for 48 h, and then the medium was changed to phenol-red free D-MEM or RPMI containing 0.1% DCC-FBS to promote estrogen-free and serum-depleted conditions. EGF-mediated reduction of apoptosis induced by serum depletion was assessed in COS-7 cells in the native state (CON) or transfected with control vector (MOCK), EGFR, EGFR and ER-wild type (EGFR/wtER), EGFR and ER-Y537F mutant (EGFR/Y537F), or EGFR and ER-Y537A mutant (EGFR/Y537A). After transfection, cells were treated with 10 n*M* EGF and cultivated for 72 h before the TUNEL assay, with calculation of the apoptosis index as before *(45)*. Data (mean ± SE) were collected from four to six independent experiments.

with calculation of the apoptosis index as before *(45)*. The results showed that cells transfected with EGFR and ER-wt, but not EGFR and ER forms mutated at tyrosine-537, had a reduced level of apoptosis as compared with appropriate controls ($p < 0.01$) (Fig. 5B).

Discussion

The activation of ER by growth factors in the absence of estrogen is a well-documented phenomenon and may play a critical role in steroid receptor signaling and breast cancer development *(8,10,17,42,46)*. The present study provides evidence for direct cross-communication between EGFR tyrosine kinase and ER and suggests that such interactions between growth factor receptors and steroid receptors may contribute to the modulation of hormone activity in a ligand-independent manner. The current findings add to a growing body of evidence that the classic ER can participate in the activation of transcription and cell proliferation by different cellular pathways.

Phosphorylation of ER at serine and tyrosine residues appears to contribute to receptor activation and, possibly, binding to DNA *(2,11,22,25,26,29,30,47)*. MAPK-mediated phosphorylation of serine residues plays a role in the activation of AF-1 in the absence of estrogen. However, to obtain full activation of the AF-1 domain, it appears that other residues, as yet undetermined, must also be phosphorylated *(8)*. Our results show that, after EGF stimulation, ER can be phosphorylated on tyrosine residues and more than one tyrosine may be phosphorylated. Site-directed mutation of ER tyrosine residues at positions 43 and 537 appears to enhance basal levels of ER tyrosine phosphorylation and promotes alterations in the time course and the level of ER tyrosine phosphorylation after treatment with EGF. Similarly, previous data have demonstrated tyrosine phosphorylation of ER after stimulation of tyrosine kinase signaling in MCF-7 cells by heregulin, a ligand for HER-1/HER-2/HER-3 receptors *(10)*. It remains to be determined what contribution tyrosine phosphorylation may make in regulating the activation of AF-1 or the interactions between AF-1 and AF-2 domains of ER.

In the present studies, EGF significantly enhanced the growth and reduced the apoptototic loss of ER-negative COS-7 cells after transfection of ER in monkey kidney cells. Under estrogen-free conditions, in vivo administration of EGF similarly mimics the growth-promoting effects of estrogen in the mouse reproductive tract *(16,17)*. In addition, in knockout mice lacking ER-α, both estrogen- and EGF-stimulated uterine growth is blocked, suggesting the importance of ER for the promotion of EGF-mediated growth *(17)*. However, with assays of ER transcriptional activity using an ERE-CAT reporter gene, the present studies demonstrated that ER is only minimally activated by EGF in the absence of estrogen, a result consistent with many earlier reports *(2,4–9)* but contrasting, in part, with one study *(48)*.

Although EGF promotes significant proliferation of cells containing ER, it does not stimulate a large increment in ERE-dependent transcription. This finding is a paradox. However, results from several recent studies suggest that cell growth and ERE-dependent transcription may not be associated. Kousteni et al. *(36)* have reported that the anti-apoptotoic action of estrogen in target cells can be dissociated from the transcriptional activity of the classic receptor, and our results appear to support this finding. Remarkably, estrogen-dependent gene transcription can be inhibited by nitric oxide, but DNA synthesis induced by estradiol is unaffected by nitric oxide, thus suggesting again that some effects of estradiol are mediated by a pathway that is not dependent on ERE-related transcription *(49)*. A discordance between ERE-dependent transcriptional activity and estrogen-dependent proliferation also led earlier investigators to propose that the two processes may be exclusive cell functions *(50)*. Collectively, these findings are consistent with the hypothesis that ER-dependent proliferation and inhibition of apoptosis may occur along a different pathway than ERE-dependent transcription (see also ref. *51*). Further studies will now be required to test this hypothesis.

Cross-communication between peptide growth factor pathways and ER may prove to be very important in modulating hormonal activity in normal and aberrant tissue. One potential cellular site for interaction between ER and EGFR may be caveolae, specialized microdomains in plasma membrane. Caveolae are thought to occur in most cell types *(52)*, although with reduced expression in breast cancer cells *(53)*. Caveolae are enriched in EGFR, and EGF treatment promotes the recruitment of multiple signaling molecules to caveolae *(52,54)*. A portion of ERs in target cells also localizes in caveolar membrane fractions *(31,55,56)*, and ER can interact with caveolin-1, a defining protein in caveolae that provides a scaffold for the assembly of signaling molecules *(57)*.

A number of studies have now documented that ER is subject to phosphorylation and activation by several peptide growth factors with consequent ERE-mediated gene expression *(5–7,12,15,58)*. Altered elements in growth factor signaling pathways, such as receptor amplification and/or overexpression, may directly influence steroid hormone action in human breast cancers *(46)*. One major problem in breast cancer management is the conversion of estrogen-sensitive to hormone-resistant malignancies after initiation of antiestrogen therapy *(59)*. The molecular basis for this hormone-independent progression of breast cancer is not clear. However, enhanced cross-communication between growth factor receptor pathways and ER during cancer progression could contribute to ER activation in the absence of hormone. This development could then result in a reduced response to antiestrogens *(46)*. Current findings indicate that EGFR plays a leading role in the progression of breast tumors *(38)*. In patients with breast cancer, prognosis is inversely correlated with overexpression and/or amplification of EGFR. In addition, an inverse correlation in the expression of ER and EGFR in breast cancers correlates with aggressiveness of the disease and with the response to endocrine treatment *(46)*. Of special significance in human breast cancer, increased signaling through the EGFR pathway also results from overexpression of HER-2, an important signaling partner for EGFR *(60)*. It is hoped that further delineation of these complex pathways in breast cancer cells will lead to the design of novel therapies that combine antigrowth factor signaling strategies with antihormone measures.

Materials and Methods

EGF and estradiol-17β were from Sigma (St. Louis, MO). ICI 182,780 (7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl] estra-1,3,5*(10)*-triene-3,17β-diol), a compound with pure estrogen antagonist activity in vivo and in MCF-7 cells in vitro *(33)*, was generously provided by Dr. Alan Wakeling (Astra Zeneca Pharmaceuticals). ER-α is a recombinant human protein (66 kDa) purified from a baculovirus expression system (PanVera, Madison, WI). The translated sequence, corresponding to Genebank entry M12674, is functionally active and binds estradiol with high affinity and high specific binding activity exceeding 5000 pmol of [^{3}H]-estradiol bound/mg receptor protein (PanVera) *(61–63)*, a finding confirmed in our laboratory (data not shown). EGFR (HER1) is purified from human carcinoma A431 cells by affinity chromatography methods *(37)*. One unit of EGFR protein transfers 1 pmol of [^{32}P]-phosphate to angiotensin-II/min at 30°C at pH 7.4 *(64)* (PanVera). [δ-^{32}P]ATP was from Perkin-Elmer (Boston, MA). Antibodies to ER and EGFR were from Oncogene Research (Cambridge, MA). Agarose-conjugated antiphosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY), and agarose-conjugated antiphosphoserine antibody *(65)* was from Sigma. Anti-EGFR agarose conjugate antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

COS-7 monkey kidney cells and MCF-7 human breast cancer cells (American Type Culture Collection [ATCC] Rockville, MD) were routinely maintained as before *(10)* in Dulbecco's modified Eagle's medium (D-MEM) and RPMI-1640 containing 10% FBS, 100 U of penicillin/mL, 100 µg of streptomycin/mL, 25 µg of gentamycin/mL, and 2 m*M* L-glutamine. At 48 h before each experiment, the medium was changed to phenol red–free D-MEM or RPMI-1640 containing 1% DCC-FBS *(66)* to promote estrogen-free conditions.

Plasmids

The plasmid, pEV7-HER1, was a gift from Dr. Ke Zhang (Amgen, Thousand Oaks, CA) *(67)*. A reporter plasmid containing a palindromic ERE and the CAT gene, termed

pEREBLCAT, was a gift from Dr. Malcolm Parker (Imperial Cancer Research Fund, London, UK) *(10)*. In brief, an oligonucleotide sequence corresponding to an ERE derived from the vitellogenin A2 promoter of *Xenopus laevis* (–331 to –295) was cloned into the *Xba*I site of pBLCAT2.

The ER expression vectors used are derivatives of pIC-ER-F *(68)* and were obtained from ATCC. Site-directed mutations of ERs were constructed by established methods *(27,69,70)* using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The following primers were used:

1. ERY537F-P1: 5' AAGAACGTGGTGCCCCTCTTTGA CCTGCTGCTGGAGATG 3'.
2. ERY537F-P2: 5' CATCTCCAGCAGCAGGTCAAAGAG GGGCACCACGTTCTT 3'.
3. ERY43F-P1: 5' CCCCTGGGCGAGGTGTTTCTGGACA GCAGCAAG 3'.
4. ERY43F-P2: 5' CTTGCTGCTGTCCAGAAACACCTCG CCCAGGGG 3'.
5. ERY537A-P1: 5' AAGAACGTGGTGCCCCTCGCTGAC CTGCTGCTGGAGATG 3'
6. ERY537A-P2: 5' CATCTCCAGCAGCAGGTCAGCGAG GGGCACCACGTTCTT 3'.

Following site-directed mutagenesis, the ER cDNAs were excised from pIC-ER-F using *Eco*RI and ligated into the *Eco*RI site of the $pCDNA_3$ (Clontech, Palo Alto, CA). Restriction enzyme digestion was used to verify directional cloning. The following vectors were obtained: $pCDNA_3$ ER-WT, $pCDNA_3$ER-Y537F, $pCDNA_3$ER-Y537A, and $pCDNA_3$ER-Y43F.

Immunoprecipitation and Western Blots

Cells were grown in 100-mm Petri dishes and maintained in phenol red–free D-MEM, containing 1% DCC-FBS for 48 h. Cell transfections were carried out with methods as before *(10)* using 40 μg of Plus Reagent, 25 μL of Lipofectamine, 2 μg of pEV7-HER1, and 2 μg of either $pCDNA_3$ ER-WT, $pCDNA_3$ER-Y537F, or $pCDNA_3$ER-Y43F per plate. At 24 h after transfection, cells were treated with 2 n*M* EGF for different time periods. After treatment, cells were immediately washed 3 times with cold PBS and homogenized in cold mild lysis buffer (20 m*M* Tris-HCl, pH 8.0; 137 m*M* NaCl, 10% glycerol; 1% Triton X-100; 20 m*M* EDTA) in the presence of 1 μg/mL of leupeptin, 1 μg/mL of aprotinin, 50 μg/mL of trypsin inhibitor, 0.4 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 m*M* sodium orthovanadate. Proteins were quantified using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL). Employing methods as before *(10)*, immunoprecipitation was done using 500 μg of total protein and 10 μL of antiphosphotyrosine agarose–conjugated antibody (clone 4G10; Upstate Biotechnology) or 2 μg/mL of anti-EGFR agarose–conjugated antibody (R-1, against receptor cell surface epitope; Santa Cruz Biotechnology), overnight at 4°C. After washing four times with mild lysis buffer, samples were resuspended in 2X Laemmli sample buffer, boiled for 5 min, and separated on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After transfer, nitrocellulose membranes were subjected to immunodetection with 1μg/mL of anti-ER monoclonal antibody (clone TE-111, directed against amino acids 302–595 of ER-α) using the electrochemiluminescence Western blotting system according to the manufacturer's recommendations (Amersham Pharmacia, Arlington Heights, IL) *(10)*.

In Vitro Phosphorylation

Studies of in vitro phosphorylation were conducted by a modification of established methods *(26)*. In brief, a concentration of 15 pmol of EGFR, purified by affinity chromatography from human carcinoma A431 cells *(37)*, was incubated in buffer containing 20 m*M* HEPES (pH 7.4) and 1 m*M* sodium orthovanadate with or without 100 n*M* EGF for 3 min at 30°C. Tubes were immediately transferred to ice and incubated for 5 min after the addition of recombinant ER-α (3.2 pmol) and 0.3% Triton X-100, in the presence or absence of 100 n*M* estradiol-17β. Then, a mixture of 4 m*M* $MgCl_2$, 2 m*M* $MnCl_2$, 10 μ*M* adenosine triphosphate (ATP), and 1 μCi (6000 Ci/mmol) [δ-^{32}P]-ATP (NEN, Boston, MA) was added, and samples were incubated for 15 min. Reactions were terminated by the addition of 2X Laemmli sample buffer and boiled at 100°C for 5 min. Proteins were separated on 7.5% SDS-PAGE gels, and after running, gels were dried and exposed for autoradiographic analysis.

CAT Reporter Gene Assays

In selected experiments, ER transcriptional activity was assessed with an ERE-CAT reporter gene. Transient transfections were performed with methods as before using the pERE-BLCAT reporter vector *(10,27)*. In brief, cells in 60-mm Petri dishes were transfected using 2 μg of pERE-BLCAT vector and 1.5 μg pEV7-HER1 in combination with 1.5 μg of $pCDNA_3$ or 1.5 μg of pEV7-HER1 in combination with 1.5 μg of $pCDNA_3$ER-WT. Then, 30 μL of Superfect reagent (Qiagen, Valencia, CA) were added per dish. Transfection was performed for 16 h in the presence of 1% DCC-FBS in phenol red–free D-MEM. At 24 h after transfection, cells were treated with vehicle alone, 2 n*M* EGF, 10 n*M* estradiol-17β, or 1 μ*M* ICI 182,780. CAT reporter assay was performed after 18 h of treatment using the CAT enzyme-linked immunosorbent assay kit from Roche Molecular Biochemicals (Indianapolis, IN). Equal amounts of protein were analyzed in duplicate for CAT activity, and data were collected from at least three independent experiments.

Cell Proliferation Assay

Proliferation assays were a modification of methods described elsewhere *(10,71)*. In brief, prior to each transfection, COS-7 cells were maintained in phenol red–free D-MEM containing 1% DCC-FBS for 48 h *(66)*. Cells were

transfected in six-well plates using Lipofectamine Plus according to the manufacturer's recommendations (GIBCO-BRL, Life Technologies) *(72)* at the following concentrations: 4 µL/well of Lipofectamine; 6 µL/well of Plus reagent; 1 µg of $pCDNA_3ER$-WT, $pCDNA_3ER$-Y537F, or $pCDNA_3ER$-Y537A expression plasmid; and 1 µg of pEV7-HER1 for a total of 2 µg of DNA per well. Duplicate wells were transfected using 2 µg of pCMVβgal/well. After 5 h of incubation, the medium was aspirated and new phenol red–free D-MEM containing 5% DCC-FBS was added. After 24 h, each well was divided into 6 wells of a 12-well plate and half were treated with 2 n*M* EGF in phenol red–free D-MEM, 1% DCC-FBS for 72 h. Cell numbers were determined by direct counts using a hemocytometer. Final data were determined from a minimum of four independent experiments.

Apoptosis Assay

Cell cultures were plated in standard media for 48 h, then changed to analyzed for apoptosis using a detection system described previously *(45,74)*. Apoptosis was assessed by a specific colorimetric detection system (Promega, Madison, WI) *(73,74)*. In brief, fragmented DNA of apoptotic cells were end labeled using a modified TUNEL assay. Biotinylated nucleotide was incorporated at 3'-OH DNA ends using terminal deoxynucleotidyl transferase. Horseradish peroxidase–labeled streptavidin was then bound to biotinylated nucleotides and detected using peroxidase substrate, hydrogen peroxide, and the stable chromogen diaminobenzidine. Using this procedure, apoptotic nuclei stained brown. An apoptotic index was estimated by the percentage of cells scored with a light microscope at ×200 *(45)*.

Statistical Analysis

In each experiment, data are presented as mean ± SEM. The data in each experimental treatment group were compared with that in the control group using a *t*-test for paired or unpaired observations as appropriate by conventional methods *(75)*, with probability values given in parentheses.

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Steroid Hormone Receptors in Target Cell Membranes

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Numerous reports of rapid steroid hormone effects in diverse cell types cannot be explained by the generally prevailing theory that centers on the activity of hormone receptors located exclusively in the nucleus. Cell membrane forms of steroid hormone receptors coupled to intracellular signaling pathways may also play an important role in hormone action. Membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation. Recent dramatic advances in this area have intensified efforts to delineate the nature and biologic roles of all receptor molecules that function in steroid hormone–signaling pathways. This work has profound implications for our understanding of the physiology and pathophysiology of hormone actions in responsive cells and may lead to development of novel approaches for the treatment of many cell proliferative, metabolic, inflammatory, reproductive, cardiovascular, and neurologic defects.

Key Words: Steroid hormone action; plasma membrane; receptor.

Introduction

The broad physiologic effects of steroid hormones in the regulation of growth, development, and homeostasis have been known for decades. Often, these hormone actions culminate in altered gene expression *(1)*, which is preceded by nutrient uptake and other preparatory changes in the synthetic machinery of the cell *(2)*. Owing to certain homologies of molecular structure, specific receptors for steroid hormones, vitamin D, retinoids, and thyroid hormone are often considered a receptor superfamily. The actions of ligands in this steroid receptor superfamily are commonly postulated to be mediated by receptors in the cell nucleus. On binding ligand, nuclear receptors associate with target genes and permit selective transcription. This genomic mechanism is generally slow, often requiring hours or days before the consequences of hormone exposure are evident. However, steroids also elicit rapid cell responses, often within seconds. The time course of these acute events parallels that evoked by peptide agonists, lending support to the conclusion that they do not require precedent gene activation *(2–5)*. Rather, many rapid effects of steroids, which have been termed *nongenomic*, appear to be owing to specific recognition of hormone at the cell membrane. Although the molecular identity of binding sites remains elusive and the signal transduction pathways require fuller delineation, there is mounting evidence that steroid action is initiated by plasma membrane receptors.

A current challenge is to determine the relation of rapid responses to steroid hormones to intermediate and long-term effects. Some questions that arise in this context include the following: Is specific membrane binding responsible merely for cellular entry of the hormone? Do plasmalemmal receptors escort ligand to the nucleus? Are the membrane binding sites coupled to rapid signal transduction systems that also act in concert with nuclear transcription factors? Are the membrane receptors identical to nuclear receptors, modified forms, or entirely different entities? This review explores these important issues. In preparing this work, more than 1200 references providing significant evidence for rapid steroid actions and for membrane forms of steroid receptors were identified. Only a fraction of these citations can be presented here, and the reader is referred to several recent reviews in this area *(3–7)*.

Estrogens

As with other steroid hormones, biologic activities of estrogen in breast, uterus, and other tissues are considered to be fully mediated by a specific high-affinity receptor in cell nuclei. Estrogens are accumulated and retained in responsive cells, and it has been commonly assumed that the steroid diffuses passively to intracellular receptors. However, estradiol is a lipophilic molecule that partitions deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of relevant receptors *(3)*. Several investigations now demonstrate that steroid hormones enter target cells by a membrane-mediated process that is saturable

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Table 1
Brief Chronology of Selected Reports Documenting Occurrence and Activity of Membrane Steroid Hormone Receptors[a]

Steroid	Year	Observation	Reference
Estradiol	1967	Elevation of uterine cAMP by estrogen within seconds	*11*
	1975	Rapid endometrial cell calcium mobilization by estrogen	*9*
Corticosterone		Binding to plasma membranes of rat liver	*108*
Estradiol	1976	Effects on electrical activity of neurons	*20*
	1977	Specific plasma membrane binding sites for estrogen	*16*
Cortisol		Electrophysiologic effects on neurons	*21*
Progesterone	1978	Induction of oocyte maturation by steroid linked to a polymer	*29*
Estradiol	1979	Increased proliferation of cells with membrane ER	*17*
	1980	Molecular properties of ERs in liver plasma membrane	*13*
Vitamin D	1981	Rapid intestinal cell calcium uptake	*109*
Progestin	1982	Specific binding to oocyte surface and role in meiotic maturation	*30*
		Steroid receptor of 110 kDa on oocyte surface by photoaffinity labeling	*31*
Corticosterone	1983	Binding to synaptic plasma membranes	*50*
Estradiol	1983	Increase in density of microvilli at endometrial cell surface within seconds	*112*
	1984	Primary internalization of ER in endometrial plasma membrane vesicles	*104*
Thyroid hormone	1985	Characterization of plasma membrane binding sites	*47*
Estradiol	1986	High-affinity binding sites in breast cancer cell plasma membranes	*26*
		Altered breast cell membrane potential, density of microvilli within seconds	*110*
Glucocorticoid	1987	Correlation between membrane receptor and apoptosis in lymphoma cells	*53*
Vitamin D	1989	Rapid activation of phospholipase C (PLC) in rat intestine	*5,14*
		Activation of calcium channels in osteoblasts	*63*
Thyroid hormone		Rapid induction of glucose uptake	*42*
Progesterone	1990	Stimulation of calcium influx in human sperm	*33*
	1991	Calcium uptake mediated by sperm cell surface-binding sites	
		Action at plasma membrane of human sperm	*34*
Corticosterone		Correlation of neuron membrane receptors with behavior in newts	*51*
Aldosterone		Rapid effects on Na^+/H^+ exchange	*111*
Glucocorticoid	1993	Antigenic similarity between membrane and intracellular receptors	*54*
Estradiol		Binding and stimulation of HER-2 membrane receptor	*90*
	1994	Activation of adenylate cyclase signaling pathways	*12*
Vitamin D		Isolation of a plasma membrane receptor from chick intestine	*88*
Aldosterone		Identification of membrane receptor in human lymphocytes	*86*
Estradiol	1995	Membrane receptor with antigenic identity to nuclear receptor	*7,78*
		Greater nongenomic responses of membrane receptor–enriched neural cells	
Androgen		Rapid increase in cytosolic Ca^{++} in Sertoli cells	*36*
Estradiol	1997	Membrane action and PLC regulation	*14*
		Isolation of membrane binding-proteins from rat brain	*81*
Vitamin D	1998	Blocking of hormone activation of PKC by antibody to membrane receptor	*65*
Estradiol	1999	Rapid Ca^{++} mobilization required for activation of MAPK	*10*
		Rapid actions in neurons from ERα knockout mice	*94*
		Reduction of membrane ER expression by antisense to nuclear ER	*80*
		Membrane and nuclear ERα, and ERβ, each expressed from single transcript	*25*
		Activation of G-proteins, IP_3, adenylate cyclase, and MAPK by membrane ER	
Androgen		Rapid activation of MAPK pathway in prostate	*37*
Progesterone		Cloning and expression of binding protein from liver microsomal membrane	*85*
Vitamin D	2000	Ligand-induced nuclear translocation of plasma membrane receptor	*89*
Estradiol		Surface receptor in endothelial cells recognized by monoclonal ERα antibody	*79*
		Interaction of ERα with regulatory subunit of phosphatidylinositol-3-OH kinase	*113*
		Rapid tyrosine phosphorylation of Raf-1 and activation of MAPK resulting in prolactin gene expression in pituitary cells	*114*

[a]More than 1200 publications on membrane steroid receptors have appeared in the past 30 yr. Of these, only representative examples are listed here. The potential roles of alternate *(25)* or variant *(56)* forms of steroid hormone receptors and other membrane-signaling molecules *(90,94)* remain to be clarified.

and temperature dependent *(3,8)*. Moreover, it is well established that estrogen can trigger in target cells rapid surges in levels of intracellular messengers, including calcium *(9, 10)* and cyclic adenosine monophosphate (cAMP) *(11,12)*, as well as activation of mitogen-activated protein kinase (MAPK) *(13)* and phospholipase *(14)* (Table 1). These data have led to a growing consensus that the traditional genomic model of estrogen action does not explain the rapid effects of estrogens and must be expanded to include membrane receptors as a component of cell signaling *(2–7,15)*.

The first unequivocal evidence for specific membrane-binding sites for estradiol-17β (E_2) was reported in 1977 *(16)*. Intact uterine endometrial cells equipped with estrogen receptor (ER), but not ER-deficient control cells, bound to an inert support with covalently linked E_2. In addition, target cells that bound could be eluted selectively with free hormone, and cells so selected exhibited a greater proliferative response to estrogens than cells that did not bind *(17,18)*. Further investigations have continued to provide compelling evidence for the occurrence of a plasma membrane form of ER and support for its role in mediating hormone actions *(3)* (Table 1).

Selye *(19)* first demonstrated that steroids at pharmacologic concentrations elicit acute sedative and anesthetic actions in the brain. However, electrical responses to *physiologic* levels of E_2 with rapid onset have since been reported in nerve cells from different brain regions *(4,20,21)*. Similarly, certain vasoprotective effects of estrogen appear attributable to membrane receptors *(15,22)*. Estrogen-induced release of uterine histamine *in situ* has long been associated with rapid enhancement of the microcirculation by a process that excludes gene activation *(2)*. Reinforcing these observations are new data detailing the role of nitric oxide (NO) in vascular regulation by estrogen. Normal endothelium secretes nitric oxide, which relaxes vascular smooth muscle and inhibits platelet aggregation. Estrogens elicit abrupt liberation of NO by acute activation of endothelial NO synthase without altering gene expression, a response that is fully inhibited by concomitant treatment with specific ER antagonists *(23)*. This estrogenic effect may be mediated by a receptor localized in caveolae of endothelial cell membranes *(24)*. Such observations require extension, because several independent cell-signaling complexes that appear to participate in signal transduction to the nucleus also associate with caveolar structures *(2,3,22)*.

Estrogen deficiency is associated with significant bone loss, and research on the potential role of membrane ERs in regulating bone mass has increased. Evidence for membrane-binding sites and acute effects of estrogen with an onset within 5 s has been reported in both osteoblasts and osteoclasts *(5,13)*. The effects of estrogens on bone homeostasis also appear to involve rapid activation of MAPK *(13)*, as has also been demonstrated in certain other target cells *(10,15,25)*.

When exposed to E_2 conjugated to fluorescein-labeled bovine serum albumin (BSA), human breast cancer cells exhibit specific surface staining *(7,26)*. Since E_2-BSA is considered membrane impermeant, these conjugates, developed primarily for use as immunogens and for affinity purification of nuclear ERs, have also been used to assess the membrane effects of estrogen. However, in light of the fact that E_2-BSA is unstable in solution, especially in the presence of cells and their enzymic products, and releases measurable amounts of free steroid *(27)*, data relying only on the use of estradiol conjugates to test for membrane effects of steroids need especially careful scrutiny. It is clear that more stable, cell-impermeant derivatives of estradiol should be developed for evaluating membrane receptors.

Progestogens and Androgens

As documented for estrogens, several physiologic effects of progestogens and androgens appear to be regulated, in part, by membrane receptors. Progesterone controls components of reproductive function and behavior. Some of these activities are mediated by interaction with neurons in specific brain regions, and membrane effects appear to be important in this process *(4,28)*. Meiosis in amphibian oocytes is initiated by gonadotropins, which stimulate follicle cells to secrete progesterone. The progesterone-induced G_2/M transition in oocytes was among the first convincing examples of a steroid effect at plasma membrane, since it could be shown that exogenous, but not intracellularly injected, progesterone elicited meiosis and that many progesterone-stimulated changes occurred even in enucleated oocytes *(29–32)*. Moreover, this process may be related to progesterone-induced increments in intracellular Ca^{++} and release of diacylglycerol species that elicit a cascade of further lipid messengers *(32)*.

Progesterone elicits rapid effects on membrane receptors, second messengers, and the acrosome reaction in human sperm *(33–35)*. Assay of acute sperm responses to progesterone in subfertile patients is highly predictive of fertilizing capacity *(35)*. Effects of the steroid, present in the cumulus matrix surrounding the oocyte, appear to be mediated by elevated intracellular Ca^{++}, tyrosine phosphorylation, chloride efflux, and stimulation of phospholipases, effects attributed to activation of a membrane-initiated pathway. Indeed, two different receptors for progesterone, apparently distinct from genomic ones, have been identified at the surface of human spermatozoa *(35)*; nevertheless, a monoclonal antibody (MAb) against the steroid-binding domain of human *intra*cellular progesterone receptor (PR) inhibits progesterone-induced calcium influx and the acrosome reaction in sperm *(35)*.

As with estrogens and progestogens, androgens promote a rapid increase in cytosolic Ca^{++} in their cellular targets *(36)*. Other effects of androgens that are not attributable to genomic activation include acute stimulation of MAPK in prostate cancer cells *(37)*. The androgen, 5β-dihydrotestosterone, induces vasodilation of aorta, which may be owing to direct action of the steroid on membranes of smooth muscle cells leading to modulation of calcium channels *(38)*. In osteoblasts, membrane receptors for androgen appear to be coupled to phospholipase C (PLC) via a pertussis toxin–sensitive G-protein that, after binding testosterone, mediates rapid increments in intracellular calcium and inositol triphosphate (IP_3) *(39)*. Of note, Benten et al. *(40)* report that testosterone elicits Ca^{++} mobilization in macrophages that lack intracellular androgen receptor (AR). These cells

express an apparent G-protein-coupled AR at the cell surface that undergoes agonist-induced internalization.

Thyroid Hormones

Thyroid hormones are well known to regulate energy expenditure and development, and membrane-initiated effects may contribute to these responses. Triiodothyronine (T_3) rapidly stimulates oxygen consumption and gluconeogenesis in liver *(41)*. T_3 also promotes an abrupt increase in uptake of the glucose analog, 2-deoxyglucose, in responsive tissues by augmenting activity of the plasma membrane transport system for glucose *(42)*. In rat heart, T_3 elicits a positive inotropic effect, increasing left ventricular peak systolic pressure, as early as 15 s after hormone *(43)*. In each tissue investigated, alterations in intracellular Ca^{++} induced by thyroid hormone appear to modulate signal transduction to the cell interior *(41–44)*.

Membrane-initiated effects of T_3 have been documented in bone cells by means of inositol phosphate signaling *(45)*, and in brain through calcium channel activation *(46)*. T_3 can also influence other cell processes, including the exocytosis of hormones and neurotransmitters *(46)*, rapid effects that may be attributable to mediation by membrane receptors *(44)*. Although uptake of T_3 can occur concomitantly with receptor-mediated endocytosis of low-density lipoprotein, and likely accompanied by carrier proteins, uptake of T_3 itself has also been reported to occur in numerous tissues by means of a high-affinity, stereospecific, and saturable process *(45,47,48)*, as found for steroid hormones *(3,8)*.

Glucocorticoids

In addition to their long-established effects on mobilization of energy sources by promoting catabolism and the induction of enzymes involved in gluconeogenesis, glucocorticoids have profound effects on neuron signaling and on induction of apoptosis in lymphocytes, phenomena that appear to be membrane-initiated events. Kelly et al. *(21)* found that glucocorticoids rapidly altered neuron-firing patterns, and many studies have verified these effects *(4,6,28)*. These molecular events lead to glucocorticoid modulation of specific brain functions, such as the rapid response of hypothalamic somatostatin neurons to stress *(49)*. Such abrupt changes in neuron polarization are reinforced by findings of specific, saturable binding of corticosterone to neuron membranes *(50,51)*. Specific, high-affinity corticosterone binding to calf adrenal cortex plasma membrane is also identified by use of the biologically active radioligand [^{3}H]corticosterone *(52)*.

Glucocorticoids also play an important role in the regulation of immune function and inflammation. In lymphoproliferative diseases, glucocorticoids are in wide use as therapeutic agents, but the cellular mechanism leading to the therapeutic effect remains unclear. In several studies using both cell lines and freshly prepared leukemia or lymphoma cells, the presence of a membrane receptor for glucocorticoids has been implicated in modulating apoptosis and cell lysis *(7,53–55)*. Moreover, in lymphocytes, the membrane-binding site is antigenically related to the intracellular glucocorticoid receptor (iGR) and may be a natural splice variant form of the intracellular receptor *(7,55,56)*. A potential parallel to the ER transfected in Chinese hamster ovary (CHO) cells *(25)* is evident.

Aldosterone and Digitalis-Like Steroids

Beyond its classic functions of promoting renal reabsorption of sodium and excretion of excess potassium, aldosterone enhances sodium absorption from colon and urinary bladder. In each tissue, the mineralocorticoid effect is owing to enhanced activity of amiloride-sensitive sodium channels. Aldosterone rapidly augments Na^+/H^+ exchange *(6,57)*. This function is Ca^{++}- and protein kinase C (PKC)-dependent but independent of nuclear receptor activation, transcription, and protein synthesis *(6,58)*. Similarly, "nongenomic" action of aldosterone has also been reported to underlie its acute effects on cardiac function and on sodium transport in vascular smooth muscle cells *(6,58)*.

Digitalis-like compounds are often forgotten members of the steroid superfamily. These plant-derived agents elicit inotropic and chronotropic effects on the heart but also affect many other tissues. Endogenous steroidal ligands, termed *digitalis-like* or *ouabain-like* factors, have been found in sera of humans and other animals with blood volume expansion and hypertension *(59,60)* and may be released from adrenal cortex *(60)*. These ligands elicit inhibition of membrane-associated Na^+,K^+-ATPase, likely the principal receptor for these agonists. It is notable that the steroid-binding domain of Na^+,K^+-ATPase and that of nuclear hormone receptors share significant amino acid sequence homology *(61)*. In addition to membrane actions of these compounds on Na^+,K^+-ATPase, ouabain-induced hypertrophy in myocytes is accompanied by promotion of Ca^{++} flux and initiation of protein kinase–dependent pathways leading, in turn, to specific changes in transcription and altered expression of early response- and late-response genes *(62)*. Thus, the biologic effects of digitalis-like compounds, long considered the exception to the concept of exclusive genomic influence, may render them more closely integrated with the steroid hormone superfamily than was previously recognized.

Vitamin D Metabolites

Membrane-initiated effects of the seco-steroid hormone, 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), are well documented in bone and cartilage. In osteoblasts, Caffrey and Farach-Carson *(63)* elucidated possible connections between rapid effects of $1,25(OH)_2D_3$, requiring milliseconds to minutes, and longer-term effects owing to gene expression. Their laboratory was the first to show activa-

tion of calcium channels by $1,25(OH)_2D_3$ *(63)*. Calcium, which can signal gene expression through multiple pathways, promotes key phosphorylation events in certain bone proteins *(5)*. Osteoblasts exhibit rapid changes in IP_3 and diacylglycerol in response to vitamin D metabolites via activation of PLC *(5,14)*. Other bone cells with rapid responses to vitamin D metabolites include osteosarcoma cells and chondrocytes *(5,64)*. The latter system is particularly intriguing because chondrocytes elaborate matrix vesicles that appear critical in bone mineralization. The matrix vesicles, which lack nuclei, exhibit specific, saturable binding of $1,25(OH)_2D_3$, especially when derived from growth zone chondrocytes *(65)*.

Other rapid effects of vitamin D occur in a variety of cell types. Muscle cells respond within seconds to $1,25(OH)_2D_3$ via several mediators that alter cardiac output in some instances, while acute activation of calcium channels in skeletal muscle promotes contraction *(5,66)*. Of note, in lymphoproliferative disease, $1,25(OH)_2D_3$ appears to prime monocytic leukemia cells for differentiation through acute activation or redistribution of PKC, Ca^{++}, and MAPK *(5, 67)*. In pancreas and intestine, activation of membrane-associated signaling pathways results in vesicular exocytosis. Pancreatic β-cells respond to $1,25(OH)_2D_3$ with enhanced intracellular Ca^{++} coupled to increased insulin release *(68)*. In intestine, $1,25(OH)_2D_3$ stimulates exocytosis of transported vesicular calcium and phosphate. These cellular events may be related to vitamin D–promoted alterations in the levels of α-tubulin *(5)*, thereby influencing assembly of microtubules and possibly providing a means for vectorial transport of absorbed ions. Several signal transduction pathways have been found to respond rapidly to exogenous $1,25(OH)_2D_3$, including activation of protein kinases and promotion of abrupt increments in Ca^{++}, but integration of these signaling cascades with the physiologic response of enhanced ion absorption remains to be established *(5,68,69)*.

Investigations with vitamin D congeners have recently indicated the potential hormonal nature of $24,25(OH)_2D_3$, once thought to represent merely the inactivation product of precursor $25(OH)D_3$. Acute effects of $24,25(OH)_2D_3$ have been observed in bone cells and in intestine; $24,25(OH)_2D_3$ also inhibits rapid actions of $1,25(OH)_2D_3$ *(5)*. This may explain why abrupt effects of $1,25(OH)_2D_3$ often fail to be observed in vivo *(70)*: normal, vitamin D–replete subjects have endogenous levels of $24,25(OH)_2D_3$ sufficient to inhibit acute stimulation of calcium transport by $1,25(OH)_2D_3$, thus providing a feedback regulation system *(69)*.

Retinoids

Retinoic acid exerts diverse effects in the control of cell growth during embryonic development and in oncogenesis. It is widely considered that effects of retinoids are mediated through nuclear receptors, including those for retinoic acid as well as retinoid X receptors *(1)*. However, other retinoid response pathways appear to exist, independent of nuclear receptors *(71)*. Cellular uptake of retinol (vitamin A) may involve interaction of serum retinol-binding protein with specific surface membrane receptors followed by ligand transfer to cytoplasmic retinol-binding protein *(72)*. In this regard, targeted disruption of the gene for the major endocytotic receptor of renal proximal tubules, megalin, appears to block transepithelial transport of retinol *(73)*. It is noteworthy that megalin may also be implicated in receptor-mediated endocytosis of $25(OH)D_3$ in complex with its plasma carrier *(74)*. In addition, retinoic acid binds mannose-6-phosphate (M6P)/insulin-like growth factor-2 (IGF-2) receptor with moderate affinity and appears to enhance its receptor activity *(75)*. M6P/IGF-2 receptor is a membrane glycoprotein that functions in binding and trafficking of lysosomal enzymes, in activation of transforming growth factor-β, and in degradation of IGF-2, leading to suppression of cell proliferation. The concept of multiple ligands binding to and regulating the function of a single receptor is relatively novel but has important implications for modulating and integrating the activity of seemingly independent biologic pathways.

Properties of Membrane Receptors for the Steroid Superfamily

Despite renewed interest in membrane steroid receptors, the physical identity of receptors with high binding affinity for ligand remains elusive. Isolation and structural characterization of these molecules remains to be accomplished. They may be known membrane components (e.g., enzymes, ion channel subunits, receptors for nonsteroid ligands), with previously unrecognized binding sites for steroids, new forms of steroid hormone receptors, "classic" receptors complexed with other membrane-associated proteins, or truly novel membrane proteins.

Estrogens and Progestogens

Efforts to isolate and purify membrane receptors that mediate rapid effects of steroids are under way in several laboratories (Table 2). Early work on purification of ER from uterus and liver plasma membranes suggested that it was a protein species with high-affinity, saturable binding specific for estradiol-17β *(16,18)*. The molecular size of solubilized receptor was in the range of intracellular ER *(18,76)*. Other work to isolate plasma membrane estrogen-binding proteins identified the 67-kDa species characteristic of nuclear receptor, but additional proteins of variant size ranging from 28 to 200 kDa were also revealed *(77)*. To determine whether membrane ER had antigenic homology with nuclear ER, Pappas et al. *(78)* used antibodies prepared to different functional epitopes of intracellular receptor and demonstrated surface labeling in nonpermeabilized rat pituitary cells by confocal scanning laser microscopy. Recent work by Russell et al. *(79)* has demonstrated, by means

Table 2
Representative Examples of Physical Properties of Membrane-Associated Receptors for Ligands of Steroid Hormone Superfamily[a]

Ligand	MW (kDa)	K_d (*M*)	Binding capacity (fmol/mg protein)	Homology with nR	Tissue	Reference
Estradiol	51–78[b]	2.8×10^{-10}	526	ND	Rat hepatocytes	*18*
	105–148[c]					
	11–67	3.6×10^{-10}	370	ND	Rabbit uterus	*77*
	67			Yes	CHO cell (ER transfected)	*25*
Progestin	110	5×10^{-7}		ND	Amphibian oocyte	*30*
	110	1×10^{-6}		ND		*31*
	28,56	6.9×10^{-8}	Variable	ND	Porcine liver	*84*
Vitamin D	65	7×10^{-10}	240	No	Chick intestine	*88*
		1.7×10^{-11}	124	No	Rat growth chondrocytes	*65*
		2.8×10^{-11}	100	No	Rat resting chondrocytes	
	36	1×10^{-8}		ND	Rat osteoblast-like cells	*87*
Aldosterone	50	1.1×10^{-8}	350	No	Pig liver	*86*
Glucocorticoids		1×10^{-7}		ND	Rat synapses	*50*
	97–150	2.4×10^{-7}	384	Yes	S-49 lymphoma cells	*55*
		5.1×10^{-10}		ND	Amphibian synapses	*51*
Thyroid hormone	145	2×10^{-9}	320	No	Human placenta	*47*
		6×10^{-10}		ND	Rat myoblasts	*48*

[a]Only representative examples of steroid-binding membrane macromolecules are presented here. Please refer to text for additional references. Homology of membrane macromolecules to nuclear receptor forms (nR) is noted; MW, apparent molecular weight; ND, not determined.
[b]High salt (0.4 *M* KCl).
[c]Low salt (0.01 *M* KCl).

of monoclonal anti-ERα, that human endothelial cells possess surface-binding sites for estrogen (see Table 1). In evaluating the source and distribution of membrane ER, target cells with expression of ERα were treated with antisense oligonucleotide to nuclear ERα to suppress expression of receptor protein *(80)*. This approach significantly reduced expression of membrane as well as nuclear forms of ER. Using an alternate method to assess receptor origin, Razandi et al. *(25)* transfected cDNA for ERα and ERβ into CHO cells, which do not normally express ER. The transfections resulted in ER expression in both nuclear and membrane fractions, suggesting that membrane and nuclear ER are derived from a single transcript. In addition, both ERα and ERβ were expressed in membranes, and both receptors were capable of activating G-proteins, MAPK, as well as DNA synthesis *(25)*. In related studies, the acute stimulation of endothelial nitric oxide synthase (eNOS) by estrogen was reconstituted in COS-7 monkey kidney cells cotransfected with ERα and eNOS, but not by transfection with eNOS alone *(23)*.

Binding molecules for estrogen and progesterone, comprising several molecular species, were isolated from brain synaptosomes by affinity chromatography and characterized by electrophoresis and Western blot *(15,81)*. Microsequencing of one E_2-binding protein indicated that the high-affinity site corresponds to the subunit of an ATPase/ATP synthase. In addition, some studies suggest that estrogen bound to sex hormone–binding globulin, a plasma protein, also binds with specificity to membrane sites recognizing the liganded transport protein *(82)*. These transport-protein interactions promote cAMP generation via the intermediacy of G-proteins. However, further characterization of receptors for such steroid:protein complexes is not available, and it must be recalled that estrogen is in noncovalent association with its plasma protein carrier and dissociates readily therefrom *(83)*.

Binding of progesterone to plasma membrane of amphibian oocytes is specific, saturable, and temperature dependent *(31,32)*. Photoaffinity labeling with the synthetic progestin [^{3}H]-R5020, followed by gel electrophoresis, revealed progestin binding to both 80- and 110-kDa proteins in oocyte cytosol, whereas only the 110-kDa R5020-binding protein was present in oocyte plasma membrane. A progesterone-binding protein (msPR) was identified in crude microsomal, rather than purified plasmalemmal, membranes from porcine liver *(84,85)*. On solubilization, a moderate-affinity site with a dissociation constant (K_d) of 69 n*M* was found, but, after further purification, affinity decreased to K_d of 228 n*M*. The final fraction contained two novel peptides of 28 and 56 kDa. Expression of msPR-cDNA in CHO cells led to slightly increased progesterone binding in microsomes, and administration of an antibody against msPR reduced rapid progesterone-initiated Ca^{++} increases in sperm *(85)*. Whether this work represents the first successful cloning

and expression of a steroid receptor associated with cell mem-branes will have to await confirmation. However, Falkenstein et al. *(85)* suggest that the native *plasma* membrane PR may actually be an oligomeric protein complex of about 200 kDa, composed only in part by 28- and 56-kDa peptides.

Glucocorticoids, Aldosterone, and Vitamin D

Progress has been made in the isolation and characterization of plasma membrane receptors for glucocorticoids, aldosterone, and $1,25(OH)_2D_3$, although at this writing, evidence of cloning of the cDNA for any of these proteins is lacking. The membrane glucocorticoid receptor (mGR) was purified from lymphoma cells by immunoaffinity binding with an MAb coupled to Sepharose-4B; the protein displayed properties similar to iGR *(55)*. Scatchard analysis of mGR yielded a K_d of 239 n*M* and B_{max} of 384 fmol/mg of protein, representing a somewhat higher number of binding sites but a lower affinity than that of the iGR. Peptide maps revealed some sequences that were unique to the membrane form *(55,56)*. Further data suggest that the mGR in lymphoma cells is a transcript variant of the iGR *(56)* (Table 2). Properties of the aldosterone membrane receptor have been analyzed by means of [^{125}I]-aldosterone photoaffinity labeling. The protein has an apparent molecular mass of 50 kDa and appears to be distinct from intracellular receptor *(86)*.

The pursuit of membrane receptor for $1,25(OH)_2D_3$ (pmVDR) by affinity isolation has been hampered by the fact that most ligand derivatives lack sufficient binding activity. Nevertheless, work by Baran et al. *(87)* indicates that the vitamin D analog, [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, does exhibit a moderate degree of specific binding to a 36-kDa protein in plasma membranes of rat osteoblast-like cells. Using sequence determination and Western blot, the labeled membrane protein was identified as annexin II, part of a family of membrane-binding proteins previously implicated in the regulation of Ca^{++} signaling, tyrosine phosphorylation, and apoptosis. Partially purified plasma membrane proteins and purified annexin II exhibited specific and saturable binding for [^{3}H]-1α,$25(OH)_2D_3$, and antibodies to annexin II inhibited [^{14}C]-1α,$25(OH)_2D_3$ bromoacetate binding to plasma membranes and also inhibited hormone-induced increases in intracellular calcium in osteoblast-like cells. Hence, these initial results *(87)* suggest that annexin II may serve as a receptor for rapid actions of $1,25(OH)_2D_3$ in rat osteoblast-like cells, but it is not known if this receptor system functions in other cell types. In independent studies, classic biochemical strategies, coupled with analyses of specific binding, were used to isolate the vitamin D membrane receptor (pmVDR) from intestinal epithelium of chicks *(88)*. Basal-lateral membranes were solubilized with detergent and subjected to ion-exchange and gel filtration chromatography. Binding activity eluted with a protein of 65 kDa, with a K_d of 0.7 n*M* *(88)*. A highly specific antibody toward plasma membrane VDR failed to recognize the nuclear receptor in Western analyses. On the other hand, a commercially available MAb generated against the "classic" nuclear receptor reacted with many proteins in nuclear fractions of chick intestine, including a band that comigrated with authentic recombinant protein, but did not detect VDR in basolateral membranes *(89)*. Antibody to the plasma membrane receptor, but not to the nuclear receptor, blocked hormonal activation of PKC. The 65-kDa protein was also observed to bind the affinity ligand, [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, and labeling was diminished in the presence of excess nonradioactive ligand *(89)*. Electron microscopic studies of duodena vascularly perfused with control media, $1,25(OH)_2D_3$, or $24,25(OH)_2D_3$ followed by immunochemical staining revealed that $1,25(OH)_2D_3$, but not control media or $24,25(OH)_2D_3$, resulted in dramatically enhanced nuclear localization of the putative membrane receptor *(89)*.

Varied Forms of Steroid Hormone Receptors in Plasma Membranes

Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies (estrogen) or variants (glucocorticoids) of nuclear receptors and, in other instances, products apparently unrelated to intracellular receptors (aldosterone and vitamin D). There is evidence for alternatively spliced transcripts of several steroid receptors, and these variant receptors give rise to proteins of different molecular size and, possibly, modified properties *(56)*. Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions, and post-translational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation, and addition of lipid anchors or other modifications, such as palmitoylation or myristoylation. Surface steroid hormone receptors may also be part of a mul timeric complex including a "classic" nuclear receptor but bound to as-yet-unidentified transmembrane proteins and coupled to membrane-associated signaling molecules *(3,7, 15,79)*. Alternatively, plasma membrane receptors for steroids may have several common structural features with, but may be distinct from, the intracellular steroid hormone receptors *(88,89)*. In the case of retinoic acid and estradiol, binding to known membrane proteins, such as M6P/IGF-2 receptor *(75)* or HER-2 receptor *(90)*, respectively, may modulate some ligand effects. Progesterone appears to interact directly with oxytocin receptor, a G-linked protein at the cell surface, and inhibits some functional effects of oxytocin signaling, thus suppressing uterotonic activity of oxytocin *(91)*. Progesterone congeners also bind with moderate affinity to γ-aminobutyrate type A ($GABA_A$) receptors that comprise ligand-gated ion channel complexes *(4, 28)*. Absence of the γ-subunit of $GABA_A$ receptor in appropriate knockout mice results in a significant decrease in

sensitivity to neuroactive steroids such as pregnanolone *(92)*. Similarly, acute vascular relaxation induced by pharmacologic levels of E_2 may be mediated by its binding to the regulatory subunit of Maxi-K channels in membranes *(93)*, thus supporting the view that some effects of steroids, at least at high micromolar concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites.

Using ERα gene knockout (ERKO) mice, Gu et al. *(94)* showed that rapid actions of estradiol at 50 nM on kainate-induced currents in hippocampal neurons still occur, and the effect is not inhibited by ICI 182,780, a pure antagonist of hormone binding to both ERα and ERβ. These investigators suggest that a distinct estrogen-binding site exists in neurons and appears to be coupled to kainate receptors by a cAMP-dependent process. However, it is important to note that alternatively spliced forms of ERα *(95)*, as well as ERβ *(96)*, can occur in ERKO mice, thus complicating the interpretation of these results. Moreover, uterine tissues of ovariectomized ERKO mice exhibit 5–10% of the estradiol binding present in wild-type uteri *(95,97)*, and the significance of these residual estrogen-binding sites in ERKO target cells is unclear. Nonetheless, further development of double ERα and ERβ gene knockouts and perfection of this new technology should prove important in deciphering the contribution of "classic" and novel receptor forms in hormone action.

In future work, it will be important to pursue isolation and characterization of constituent proteins from homogeneous plasma membranes prepared in the presence of proteinase inhibitors *(18,76,98)*. Verification of their purity should be confirmed by use of a balance sheet for enzyme or other membrane markers *(18,76)*. Screening for activity of receptor would benefit from the use of independent approaches, such as ligand binding with radio- or photoaffinity-labeled steroids and immunoassay directed toward known intracellular receptors *(15,31,55,78,86)*. These several approaches may detect membrane receptors originating from a transcript other than that of intracellular receptor. As with the mixed steroid hormone–binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of receptor proteins for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Our efforts to understand ligand-receptor interactions are often limited by simplistic "lock-and-key" models that may not accurately reflect the true state of complex molecular signaling cascades. Study of the molecular organization of several neurotransmitter receptor families has already shown that extraordinary biologic variability occurs, with multiple "keys" and multiple "locks" sometimes involved in ligand-receptor recognition *(99)*. We must consider the existence of similar high-affinity, but possibly multivalent and multifunctional, receptors in the steroid hormone superfamily *(75,91–93)*.

Perspectives

Ever since the discovery of chromosomal puff induction by ecdysone, cell regulation by steroid hormones has focused primarily on a nuclear mechanism of action. However, even the venerable steroid hormone ecdysone elicits rapid membrane effects that may facilitate later nuclear alterations *(100)*. Indeed, membrane-initiated responses appear to be the cell's earliest response to steroids and may be prerequisite to subsequent genomic responses (*2,3,7, 10*; see also Fig. 1). Coupling of surface membrane, cytoplasmic, and nuclear responses may offer a progressive, ordered expansion of initial signal. Accordingly, the terms *genomic* and *nongenomic* may not accurately define such a response continuum *(101)*. Future investigations should focus on potential interactions of membrane and nuclear steroid receptors that may promote activation of transcription and other specific hormonal responses. Molecular details of cross-communication between steroid and peptide receptors are also beginning to emerge *(3,98)*, and membrane steroid receptors may be in a pivotal location to promote convergence among diverse signaling pathways (Fig. 1). Indeed, the consequences of steroid hormone recognition at the outer cell membrane of target, but not nontarget, cells are shared by numerous other classes of regulatory molecules (cf. ref. *102*), including peptide hormones, neurotransmitters, drugs, plant lectins, mitogens, and antibodies *(3)*. Although the agonists are manifold, the signaling mechanisms are few. Primary signal recognition at the surface would be fleeting, but the mutual specificities and affinities are high, and thus sufficient for setting the appropriate signal transduction chain in motion. However, until the current surge of renewed focus on this problem, identification of these instantaneous triggering interactions for steroid hormones has accumulated relatively slowly, having been limited by technical and microanalytic barriers that are now being surmounted.

Ligand-receptor interactions depend on an extensive array of extracellular and intracellular partners to localize to membrane microdomains, recruit signaling molecules, and trigger intracellular signaling pathways. As the consequences of surface interactions are analyzed in greater depth, it will be important to evaluate further the biologic role of rapid internalization of steroid-binding sites from plasma membranes via endocytotic-lysosomal pathways *(2,3,88,101,103–105)*. These membrane-initiated events may involve cytostructural elements or scaffold proteins that contribute to signal propagation to the nucleus and the nuclear-protein matrix (*2,101,104–107*; Fig. 1). Thus, antibodies specific to intestinal membrane VDR reveal a vitamin D–induced redistribution of membrane receptor, a protein that appears distinct from intracellular receptor, to the nucleus within 5 min of binding ligand *(89)*. It is unknown whether the membrane receptor has inherent DNA- or coregulator-binding capacity to alter transcription; alter-

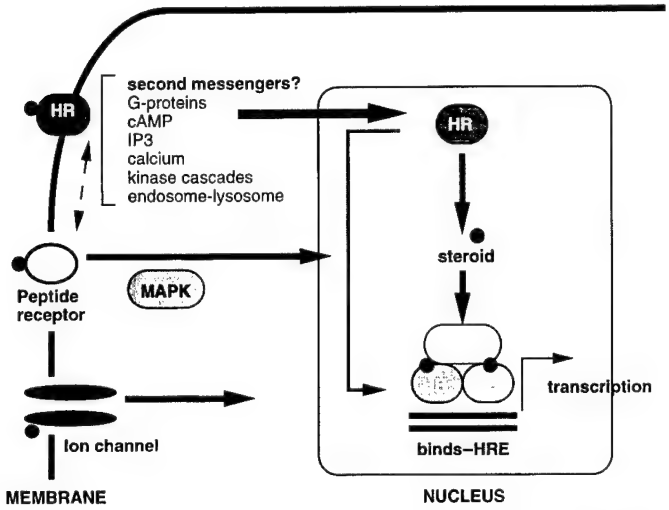

Fig. 1. Postulated mechanism of action of steroid hormones (black circles) in target cells with steroid hormone receptor (HR). In most current models, steroid binding to HR is believed to promote alterations in receptor conformation favoring enhanced association with coactivator proteins and with specific hormone-responsive elements (HRE) in the nucleus, leading, in turn, to initiation of selective gene transcription. However, the latter model fails to account for numerous, rapid cell responses to steroid treatment (see Table 1 and text). These deficiencies in the genomic model of hormone action require integration with the latter observations. In the model shown here, steroids may also bind to a membrane HR, with potential for promotion of hormonal responses via a complementary pathway that may cross-communicate or interact directly with the genomic mechanism. As noted in the text, membrane HR may be known molecules (kinases, ion channels, other receptors) with previously unrecognized binding sites for steroid, new isoforms of HR in membranes, "classic" forms of HR complexed with other membrane-associated proteins, truly novel membrane proteins, or a combination of these. Available evidence indicates that liganded membrane HR may affect one or more of several pathways, including modulation of ion channels, leading to enhanced flux of ions, notably Ca^{++}; interaction with peptide membrane receptors; and activation of G-proteins, nucleotide cyclases, and MAPK, with resultant increases in their catalytic products (see Table 1). These membrane interactions may promote phosphorylation of HR itself via steroid-induced or ligand-independent pathways. The intricate array of physiologic responses of cells to steroid hormones may occur as a consequence of a synergistic feed-forward circuit in which steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of HR (Table 1). Active reconsideration of the unqualified genomic model of nuclear receptor action is ongoing, and the probable importance of alternate signaling pathways elicited by surface recognition is now increasingly evident.

natively, it could serve to shuttle ligand to the nuclear-localized fraction of receptor. As has frequently been noted from these laboratories (cf. ref. *105*), the cellular mechanisms governing the further transport and targeting of signaling molecules are powerful avenues of current investigation.

Many issues remain to be resolved for fuller understanding of the biologic actions of steroid hormones. Foremost among these is the structural characterization of membrane steroid hormone receptors. It is now abundantly clear that the nuclear receptor–mediated mechanism as the sole means by which steroid hormones act is incomplete *(2,3,5,7,15, 107)*. It is likewise unmistakable that membrane effects of steroid hormones represent an established phenomenon that is by no means to be construed as alternative to the genomic pathway, and that demands continued investigation. Indeed, the chain of membrane-initiated events is helping to account for the relatively prolonged, apparent silence between the capture of the hormone at the surface of its preferential target and the eventual outcome in augmented genomic activities. In challenging the dogma that steroid hormones act exclusively via intracellular receptors, the membrane receptor experiments reviewed here provide a persuasive paradigm for a potentially new class of drugs for human therapy. The clinical use of steroid hormone agonists and antagonists has substantially changed the course of many hormone-related diseases, but side effects of many agents currently in use are also significant. In-depth analysis of the relative contributions of nuclear and membrane-initiated activities in steroid receptor biology may lead to the development of pharmaceutical agents that exert differential activities in the two pathways, thus favoring more selective drug delivery and promoting the emergence of novel approaches for treatment of many cell metabolic and proliferative defects.

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Plasma Membrane Receptors for Steroid Hormones: Initiation Site of the Cellular Response

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GLOSSARY

Genomic A process related to gene transcription and its regulation.

Nongenomic A process independent of RNA transcription.

Organelle An intracellular, membrane-bounded compartment , *e.g.*, mitochondrion, Golgi, lysosome, endoplasmic reticulum with membrane-bound ribosomes, nucleus, each with specialized functions, reflecting division of labor within cells.

Receptor-mediated endocytosis Cellular entry of **agonist** via a specialized region of the cell where **receptor** molecules, capable of specifically binding hormones, are localized. Such a region may also be rich in specialized proteins, such as **caveolin**. Induced invaginations may be pinched off from the outer membrane and become **endosomes** – vesicular **channels** for signal transduction.

Signal Transduction A signal is a message relayed from one site to another, in the molecular language of the cell. The *primary* signal, in the hormonal context, originates from binding of the **agonist** (active agent, the hormone) to **receptor protein** at the surface of the **target** cell. Signal transduction involves message conversion (translation) from one molecular "**language**" to another, "read" elsewhere in the cell –*e.g.*, surface interactions on hormonal impact lead to abruptly altered intracellular levels of substances with catalytic activities, such as Ca^{2+}, cyclic nucleotides, and phosphokinases (which shuttle phosphate between critical proteins, altering their structure and behavior). Thus, signal transduction, like a molecular relay, advances the hormonal message, both temporally and spatially, among the cell organelles, like a lighted fuse, progressing toward output at the terminal.

Steroid A family of lipid structures related to the parent substance, cholesterol, which is modified by enzymes in certain tissues that synthesize highly active products with hormonal functions, such as estrogen and progesterone in ovary, testosterone in testis and cortisol in the adrenal cortex (see Table I).

I. Introduction

It seems axiomatic that mutual recognition between an agonist in the extracellular fluid and the responsive cell must take place at the surface membrane that constitutes the dynamic boundary between them. This fundamental process, first

envisioned in the immunologic context by Paul Ehrlich (Fig. 1), is shared by hormones of varied structure, lipid as well as peptide. This concept is supported by a large body of evidence that has brought into harmony previously divergent views of the significance of the chemical nature of the agonist in the chain of secondary mechanisms that stem from the all-important primary step of selective interception of hormone by receptor. Examples of the lines of evidence and criteria for identifying the selectivity, specificity, and affinity of such interaction for the several steroid classes with specialized protein components of the target cell surface will be presented.

Although the cellular actions of steroid hormones were once postulated to be regulated exclusively by receptors in the cell nucleus, thus permitting selective transcription after ligand binding, this genomic mechanism generally requires hours or days before the effects of hormone exposure are evident. In addition to the latter pathway, steroids also elicit rapid cell responses within seconds of administration. The time course of these acute events lends support to the conclusion that they do not require new gene transcription. Rather, many rapid effects of steroids, termed 'nongenomic', appear to be due to specific recognition of hormone at the cell membrane. Hormone-receptor interactions at the surface membrane can initiate a cascade of signaling events that may regulate many cellular functions, both acute and prolonged.

Subsequent entry of steroid hormone into its target cells can be astonishingly swift and requires special strategies to demonstrate its temporal dissociation from binding proper. In some cases, receptor-mediated entry appears to be closely followed by partition into the several intracellular compartments. The available means of such ultrarapid penetration and dissemination will be outlined, as will the potential significance of sequential translocation in the overall cellular response. Accordingly, the proportion of total receptor, localized at a given moment in any cellular compartment, whether plasmalemmal, cytostructural, or nuclear, reflects the metabolic history of the receptive cell.

Finally, it is the purpose of the present article to survey the transduction mechanisms available to such a cell for amplifying and extending the impact of initial surface perturbation by hormone capture. Through such means of communication of the primary hormonal signal can the resultant structural and functional modulations of the several intracellular compartments, including the nuclear, be coordinated into the totality of the cellular response.

II. Supramolecular Organization of the Surface Membrane and Occurrence of Steroid Receptors

Steroid uptake in cells may occur by passive or facilitated diffusion across the plasma membrane or by one of several endocytotic mechanisms. Biophysical studies demonstrate that most steroid hormones are lipophilic molecules that partition deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of receptor proteins. However, these agonists also appear to enter target cells by a membrane-mediated process that is saturable and temperature-dependent.

Membrane Models : From Fluid Mosaic to Lipid Rafts and Signaling Platforms. To understand the nature of steroid receptor association with cell membranes, it is important to consider current concepts of supramolecular organization of the membrane (Fig. 2). The present view of the lateral organization of plasma membrane constituents has been revised significantly from the original fluid mosaic model, wherein membrane proteins were considered to diffuse freely in a sea of lipid, above a critical temperature of 15° C. With the wide array of molecules known to interact rapidly in receptor signaling, it is difficult to imagine how specific signal transduction could occur if components moved randomly in the lipid bilayer. Rather, new findings suggest the existence of macro- and micro-domains of the membrane that serve to concentrate key signaling molecules for efficient coupling to effectors. The concept of a 'signaling platform' has been advanced to characterize a structure in which many different membrane-associated components are assembled in a coordinated fashion.

Evidence now indicates that plasma membrane microdomains termed 'lipid rafts' arise from the phase behavior of lipid components. In the fluid bilayer of the membrane, different lipid species are asymmetrically distributed over exoplasmic and cytoplasmic leaflets of the membrane. In particular, long, saturated acyl chains of sphingolipids cluster in the presence of cholesterol to form a liquid-ordered phase, resistant to detergent solubilization. Saturated acyl chains of glycosylphosphatidylinositol (GPI)-anchored proteins, as well as transmembrane proteins and certain tyrosine kinases, can also occur within these lipid domains. Raft association may concentrate receptors for interaction with ligands and effectors on either side of the membrane, thus facilitating binding during signaling and suppressing inappropriate crosstalk between otherwise conflicting signal transduction pathways.

Endocytotic Adaptations. Caveolae, literally 'little caves', are more specialized raft microdomains that also concentrate and assemble components of several signal transduction pathways (Fig. 2). These membrane structures can be invaginated, flat within the plane of the membrane, detached vesicles, or fused together to form grape-like structures and tubules (Fig. 3). Like lipid rafts, caveolae are rich in cholesterol and sphingolipids, but, unlike rafts, they are lined intracellularly with clusters of caveolin protein, a cholesterol-binding molecule that contributes to membrane lipid organization. The growing list of caveolae-associated molecules constitutes a 'who's who' of cell signaling, including receptor tyrosine kinases, G-protein-coupled receptors, protein kinase C, components of the mitogen-activated protein (MAP) kinase pathway, and endothelial nitric oxide synthase (eNOS). In one such example, sub-populations of estrogen receptors are localized to caveolae in endothelial cells, and, in plasma membrane caveolae isolated from these cells, estradiol directly stimulates its receptors which are coupled to eNOS in a functional signaling module to regulate the local calcium environment and blood vessel contractility.

Clathrin-coated pits are independent membrane invaginations, decorated intracellularly with the protein clathrin. They function in endocytosis of nutrients and certain receptors, such as receptor-mediated uptake of low-density lipoprotein-

cholesterol complexes, and also play an important role in signal transduction. Some agonists may be internalized via either clathrin-coated pits or caveolae, with one pathway apparently providing a default entry mechanism for the other under certain conditions.

Raft-dependent signaling is often coupled with endocytotic uptake mechanisms involving rafts as well as caveolae. Also important in this scheme is the actin cytoskeleton, considered to provide constraints for lateral mobility of lipid microdomains and to function in endocytotic trafficking. Endocytosis itself is a diverse set of processes, which promote internalization of specialized regions of plasma membrane as well as small amounts of extracellular fluid (Fig. 3). The best understood form of endocytosis occurs at clathrin-coated pits and involves clathrin and the dynamin GTPase, which promotes pinching-off of the endocytotic vesicle. Caveolae also play an important role in potocytosis, a mechanism for uptake of small molecules across plasma membrane. Finally, some cell types can internalize larger amounts of fluid by macropinocytosis or particulates by phagocytosis (Fig. 3). In most cells, internalized materials are first delivered to early sorting endosomes, which may mature into or be transferred to late endosomes, and, ultimately, to lysosomes. The potential role of the ubiquitin-proteosome pathway in this process remains to be determined.

Steroid Receptor Variability. The precise nature of the association of steroid receptors with plasma membrane remains elusive, primarily because full structural characterization of these molecules is incomplete. The task of identifying these membrane-associated steroid receptors is made more challenging by the recent detection of multiple transcript variants of classical 'intracellular' steroid receptors, and, in the case of estrogen receptor-α (ER-α), by discovery of a structurally-related estrogen receptor form, termed estrogen receptor-ß (ER-ß), that is the product of a different gene. Both ER-α and ER-β gene products are expressed in membranes, and both receptors are capable of activating acute and late phases of cellular responses through activation of signal transduction cascades.

Estrogen receptor (ER) from target cell plasma membranes is a protein species with high-affinity, saturable binding specific for estradiol. In addition, antibodies to nuclear ER-α recognize surface sites, suggesting that membrane ER has antigenic homology with nuclear ER. Indeed, recent work reveals that membrane and nuclear ER may be derived from a single transcript. Likewise, properties of membrane glucocorticoid receptors closely resemble those of the intracellular receptor. On the other hand, properties of the aldosterone receptor, as well as those of the plasma membrane receptor for $1,25(OH)_2$vitamin D_3 suggest that membrane receptors for these steroids may be distinct from their 'classical' intracellular counterparts. Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies or variants of nuclear receptors and, in other instances, products apparently unrelated to these.

Steroid receptors in membranes may also be contained in multimeric complexes with other transmembrane molecules coupled to specific signaling cascades (Fig. 2). In the case of retinoic acid, binding to known membrane proteins, such as mannose-6-phosphate/IGF-II receptors, may occur. Likewise, progesterone congeners bind with moderate affinity to γ-aminobutyrate type A ($GABA_A$) receptors that comprise ligand-gated ion channel complexes, and pharmacologic levels of estradiol bind with regulatory subunits of independent ion channels in membranes, thus supporting the view that some effects of steroid hormones, at least at high concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites. Finally, despite subtotal ER-α gene knockout, some rapid actions of estradiol still prevail. As with the mixed steroid hormone-binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of receptor proteins for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Nevertheless, available evidence suggests that a finite portion of cellular steroid receptors is associated with signaling platforms in specialized microdomains of the plasma membrane.

III. Specific Binding of Steroid Hormones to Surface Membranes of Responsive Cells

As postulated by Ehrlich in the Croonian Lecture to the Royal Society more than a century ago, the outer surface of a responsive cell is equipped with specialized components, which exhibit exquisite discriminatory capacity toward potential agonist when molecular conformations are mutually complementary (*cf.* Fig. 1). Indeed, in evolutionary terms, steroid recognition at the surface membrane appears to have been the primary response pathway of the primitive cell. In plant cells, the only known response pathway to steroids is via a membrane-associated receptor that regulates numerous functions in the intracellular economy, including growth and development. In the case of steroid hormones which influence the functions of eukaryotic cells, the fact that such receptor molecules are poised to extract agonist from its plasma protein carrier is directly attributable to primary evidence for *non-covalent*, and thus, reversible, steroid:protein interaction. This property forms the basis for competitive displacement of ligand by excess, or by conformationally-competent congeners.

The concept of specific membrane-associated binding sites for steroid hormones has been supported by rigorously controlled observations from many independent laboratories. Evidence is now available for the extended steroid family, which includes the retinoids, thyroid hormone, and digitalis-like steroids (*cf.* Table I). The methodologic approaches have also been broad. Representative examples of several of these approaches for estrogen are presented in Figures 4-6. However, comparable observations are available for other members of the steroid family, especially adrenocortical steroids and vitamin D metabolites (Table II). Thus, from physical, ultrastructural, immunologic and molecular probes, as well as direct kinetic analyses of specific binding of isotopically-labeled steroid to the surfaces of isolated target cells or to their purified plasma membrane

(Table I) (Fig. 4) (Fig. 5) (Fig. 6) (Table II)

fractions, a large body of evidence now supports this view. Such membrane proteins constitute a fraction of total receptor molecules available at any given moment in the cellular target and have occasionally been overlooked when methods of sufficient sensitivity were not utilized and when signal-to-noise ratio was not taken into account. Especially instructive data are now available for pinpointing the surface orientation of specific receptor proteins for given steroid hormones at their cellular targets (Figs. 4-6). Recent ultrastructural studies have revealed extranuclear immunoreactivity for ER-α associated with membrane sites along dendritic spines and axon terminals of neurons (Fig. 6). Moreover, Fig. 5 reveals incipient receptor-mediated endocytosis in Hep G2 cells. These modern findings confirm the observations of Williams and Baba in 1967, at which time they reported, using electron microscopy and admitted excess of labeled steroids, that [^{3}H]-aldosterone and [^{3}H]-cortisol associated with plasma membranes of their respective target cells. It is uncanny that report of abrupt stimulation of membrane-associated adenylate cyclase activity by physiological levels of estrogen appeared in the same year, but these data, as fine red wine, required many years of aging before appealing to the taste of the wider scientific community.

Presently, there has been intensely renewed interest in documenting specific steroid binding with target cell membranes, and current extensions of these data are ongoing. One salient fact emerges from the combined observations, namely, that *there is a striking parallel between the initial encounter of steroid, as well as peptide, agonist with the surface of its responsive cell.* Such function, critical to unfolding of an orderly sequence of succeeding events through receptor-mediated coupling to further metabolic signals (see below), is also shared by many other regulatory agents, including those that promote growth and development of their target cells, such as the phytohemagglutinins in transformation of small lymphocytes, and, indeed, cytokines generally.

It is important to note emerging data, which suggest that different structural conformations of a given steroid hormone may act as specific agonists for selected cellular response pathways. For example, it is suggested that 1α,25(OH)$_2$ vitamin D$_3$ produces biologic responses through two distinct receptors, one predominant in the surface membrane and the other in the cell nucleus, respectively, which are able to recognize different shapes of the conformationally flexible molecule. Accordingly, the functional significance of agonist:receptor interactions at the target cell surface lies in the potential for selective pathway engagement for propagation of this primary signal.

IV. Consequences of Receptor Occupancy: Activation of Signal Transduction Pathways

Repercussions from the cell surface may be communicated to the farthest reaches of cell structure and function, including the transcriptional events that will eventually unfold in the nucleus. Over some decades, manifold activities, which are amplified over the relatively prolonged intervening period, have been documented. In the case of estrogen, which has received the most

attention among the steroid hormones in this regard, the time-course of such events encompasses several orders of magnitude, leading to its general description as a continuum (Figs. 7 and 8). A similar temporal distribution pattern prevails for responses to glucocorticoids and vitamin D metabolites (Table II).

Propagation of the minimal information from the primary capture of hormone at the cell surface, through an orderly cascade of intermediary reactions in other compartments, and, ultimately, to differentiation or division of the cell so mobilized, begins through recruitment of virtually instantaneous and closely-linked processes within the affected membrane and in its immediate subplasmalemmal environment. Receptor-mediated signal transduction responses have been identified for essentially all the steroid hormones (Table III).

An Orderly Cascade. It is significant to note the time-course of these cellular activities, beginning with the earliest indications of membrane perturbation, which occur within seconds or less, such as the nucleotide cyclase reactions. Here, again, is a significant example of mechanism shared by steroid and peptide agonist. It is particularly well illustrated in neural responses. Acute alterations in Ca^{2+} and in Na^{+}/K^{+} flux are likewise rapid and occur within wide differences in agonist and end-organ. Abrupt changes in phosphorylation mechanisms, some of which are Ca^{2+}-dependent, are also recruited. Many of these changes in the cytoplasmic microenvironment, in turn, have profound effects upon enzymic reactions and upon cytologic structure, with special reference to protein folding. Thus, amplification of primary hormonal signal is achieved with great conservation of energy and without further input of mass, through a limited number of receptor-mediated transduction mechanisms, linked, in part, through heterotrimeric G-proteins that are integral to plasma membrane. These remarkably conserved features of hormone action are covered in depth elsewhere within these volumes.

In the case of some hormonal responses, interaction at the surface membrane may itself be sufficient to elicit an alteration in cell function. For example, estradiol can directly stimulate protein kinase C activity in membranes isolated from chondrocytes, and the steroid also modulates calcium-dependent eNOS activity associated with its receptor in isolated plasma membranes from endothelial cells. Moreover, estrogens may enhance growth of mammary tumor cells, largely independent of estrogen-responsive element (ERE)-dependent transcription, by stimulating membrane-associated MAP kinase pathways. Ligand-independent activation of steroid hormone receptors also occurs and may represent a more primitive response pathway, whereby cross-communication with peptide signaling systems in the cell can directly modulate the activity of steroid hormone receptors. For example, estrogen receptor can be activated in the absence of estradiol through phosphorylation by EGF-stimulated MAP kinase. Any comprehensive model of steroid hormone action must account for these important cellular interactions.

Transitory Alterations in Cellular Architecture and Translocation of Receptor. Among the numerous, acute responses to estrogen recognition in uterine preparations are brief, transitory alterations in cellular architecture, beyond the clear evidence of regional perturbation (*cf.* Fig. 7), and include incipient vesiculation within the membrane itself (*cf.* Fig. 5). These cytoplasmic responses occur within seconds or less, and comprise striking transitory reduction of arrays of microtubules and microfilaments. Indeed, there is considerable evidence that microtubules and the actin cytoskeleton of the cell play an important role in endocytotic trafficking and concomitant signal transduction. In some cases, such remarkable early modifications of target-cell structure may themselves play a key role in signal propagation by serving to modulate the relative viscosity of the medium in which hormone: receptor [H:R] complex is translocated toward, and into, the nuclear compartment.

The microtubular apparatus, with its arboreal array spanning the sub-plasmalemma and perinuclear/ Golgi regions, has been implicated even more directly in the translocation mechanism for the vitamin D_3 receptor in mouse osteoblasts, as well as in the case of cellular targets to glucocorticoids. There are now clear indications that, at least for some steroid hormones, a significant portion of the hormone:receptor complex occurs in vesicular form (*cf.* Figs. 5-7), with the potential for fusion with other organelles.

Sequential distribution of hormone. Because of the extreme speed of entry, the temporal association of steroid hormone with a surface receptor and its ensuing distribution in target cells has been difficult to demonstrate without appropriate precautions to eliminate nonspecific membrane-perturbing influences. These precautions include strict omission of serum and phenol red from media, use of incubation temperatures at 23° C. rather than the customary 37° C., but not below 15° C., when lipid components of membranes assume a rigid conformation, and, above all, sampling at very short intervals. Indeed, because of lack of appreciation by many investigators of these precautions, cumulative evidence of such association had been overlooked by some for decades.

An especially telling analysis of the [^{3}H]estradiol-17ß translocation mechanism is available, using analytical cell fractionation at progressive time periods, beginning within 10 seconds of exposure. Estradiol-17ß interacts specifically with membrane proteins in uterine cells and undergoes rapid internalization in nanometer-sized endocytotic vesicles resulting in delivery of a portion of the steroid hormone and its associated receptor protein to the cell nucleus and nuclear protein matrix. Quantitative analyses of the postnuclear supernatant prepared from uterine cell homogenates incubated under the strictest estrogen-free conditions indicates that a significant portion of specific estrogen-binding sites is internalized from plasma membranes in vesicular form. Concomitant with a decline in plasmalemmal and presumptive endosomal fractions, a

significant amount of labeled hormone occurs in Golgi and lysosomal compartments before the peak in nuclear accumulation. These observations demand further pursuit with due regard for the scrupulous techniques required.

V. Membrane Signaling and the Cellular Response to Steroid Hormones

Compartmentation in the Cellular Economy. Without some form of communication between the events at the cell surface and the relatively remote nucleus, separated as it is from all else in the cell by a double membrane, the coordinated response of growth or differentiation could not be achieved. Indeed, there is rapidly growing evidence that there is close synergism between the receptor-mediated, virtually instantaneous activities at the plasma membrane and their considerably delayed effects within the nucleus. Clearly, mechanisms exist for transfer of information, as well as matériel, between the two major cell compartments.

Separation of potential reactants by structural barriers of variable degree of penetrability is a primitive yet thermodynamically efficient means of maintaining a poised system. Such a system is capable of rapid responses to changes in its environment if specialized surface components can detect and capture minute amounts of specific regulatory agents. In the fullest sense, the steroid-hormone target cell is just such a system.

The initial stages of the primary response may constitute physicochemical alterations in conformation that promote propagation of signal with the speed of the phase changes reminiscent of the child's game of cat's cradle. The information gap between the cell surface and the boundaries of the other cellular organelles, most notably, the nucleus, is then closed, with variable rates of speed, by a chain of ordered secondary reactions originating from the coupling of liganded receptor to other cell signaling proteins (*cf.* Figs. 8 and 9). (Fig. 9)

Now, under certain conditions, these transduced responses, in a closely coordinated system of interdependent pathways, forward the expanded signal toward the nucleus and the enhanced genomic activities to come. There have been significant advances in demonstration of hormone and/or receptor in vesicular form, in close perinuclear array at very early times after surface binding and before substantial concentrations occur within the nucleus. The specific means by which the formidable nuclear barrier is crossed have not yet been identified in the hormonal context. However, there are strong indications of organellar intervention and membrane fusion in hormone:receptor complex transport and, in specific cases, delivery through compound lysosomal pathways. At the same time, the ionic, enzymic, and energy-generating functions, recruited in coupled fashion in the cytoplasm, prepare the responsive cell for its expanding metabolic requirements.

The genomic hypothesis of steroid hormone action has generally prevailed as the exclusive mechanism since 1961, the year in which the seminal concepts of Jacob and Monod electrified the scientific community. As will be presented in other

chapters of this Series, in the interval to the present, extraordinary accomplishments by a broad array of molecular biologists have extended and clarified the details of these concepts for the late nuclear repercussions of a number of steroid hormones at their cellular targets, while unfortunately overlooking the well-documented responses attributable to signal at the cell surface. It was inevitable that the emphasis upon the critical and novel activities triggered at the nuclear level would overshadow the parallel observations being made on receptor-mediated signals emanating from the primary recognition site, the cell membrane.

Recent advances have now permitted greater focus upon the acute signals and their systematic transduction. This renewed outlook restores the necessary balance to our understanding of steroid hormone action, and integrates the contribution of each set of functions into a more complete whole (Fig. 9). Moreover, in the case of some hormone responses, the primary interaction at the surface membrane may be sufficient of itself to elicit a cascade of intracellular signals to specifically alter cell function.

Direct, Membrane-Initiated Responses Seemingly Uncoupled from the Cytoplasmic Cascade: The Dual Functions of Surface Receptor Activation. What is not yet clear, except under the special circumstances noted below, is the question of the inexorability of the full sequence of transduction steps from cell surface recognition to genomic activation, and, thereby, to growth or differentiation. Is there a briefer, less extensive pathway–essentially only an abbreviated sequence–that leads to altered cell functions, including those related to the increase in number of osmotically-active particles at a very early stage of structural changes in membrane 'permeability'? As noted briefly above, one such example that comes immediately to mind is the localized liberation of nitric oxide, itself secondary to an instantaneous surge of Ca^{2+}, which occurs in response of endothelia to estrogen; these coupled events result in rapid vasodilation, thus clearly by-passing the hours-long, metabolically expensive transduction pathway leading to nuclear arousal. Such a truncated pathway may parallel only one or two early steps of the full sequential transduction route. The local effects of estrogen on electrophysiological activities of neurons is another obvious case in point. In the instances noted, there is distinct evolutionary advantage to such a short-cut. In fact, there are circumstances currently being identified, in which the two response-sequence stages, full and partial, coexist side by side, thus supporting acute, as well as delayed, responses to surface signal, independently and in parallel.

Accordingly, the functions of the surface receptor are twofold. Both lead to coordination of the activities of more distal organelles. One such function is *complementary* to the more remote and time-delayed events at the genome, through communication of information, both signals and matériel, from the extracellular environment. The second function *supplements* the more delayed and metabolically demanding activities at the genome, through short-cut of the latter. Instead, signals, transduced from receptor engagement of steroid ligand at the external cell surface, are themselves converted, independently of genomic activities, into sharply immediate and readily reversible stimuli, such as those eliciting changes in

nervous activities and vasomotor functions – these, of evolutionary significance for survival. These dual capacities of surface receptor activation underlie perfect adaptation of the receptive cell to the processing of information from its external environment on two independent/interdependent tracks: acute and more prolonged (Fig. 9).

VI. Summary

Rigorously-controlled experimental data, originating from the work of investigators dispersed world-wide, demonstrate that steroid hormones are first intercepted by specialized proteins associated with the surface membrane. Recognition occurs by features of mutual structural conformity, as predicted from principles of physics and chemistry applied to cell biology. For estrogen and glucocorticoid, such receptor molecules at the cell surface have been found to share homology with the nuclear forms, whereas this is not the case for vitamin D metabolites, aldosterone, or thyroid hormone in the limited numbers of tissues examined. Such information is generally lacking for other members of the steroid superfamily.

Capture of steroid agonist from the extracellular fluid is attributable to the competitive advantage of the cellular receptor, for its affinity for ligand is several orders of magnitude higher than that of the carrier proteins in the circulation. This demonstrates that agonal:receptor interaction is reversible, and conforms to the laws of mass action.

Once effective concentrations of hormone are so bound, the cell surface undergoes virtually instantaneous but transitory structural reorganization. These primary interactions may trigger a cascade of specific cellular responses. Thereafter, a portion of the hormone:receptor complex is internalized, generally within seconds or less, through one or more endosomal mechanisms.

Communication and coordination among the several specialized cellular organelles of the targeted cell is achieved by signal transduction processes that propel the hormone:receptor complex or other specific membrane-associated signaling partners toward and into the nucleus. These combined activities are succeeded by the late stages of the response continuum at the genomic level. The outcome is the totality of response in the context of the whole cell, through synergic functions of its organellar constituents.

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LEGENDS

Figure 1. Diagrammatic representation of the "side-chain" theory to illustrate Ehrlich's concept of specific recognition sites at the cell surface. 1. Complementarity of agonist and receptor. 2. Specific and reversible binding of agonist only to its own receptor. 3. The bound form of receptor is unavailable for providing negative-feedback toward its own biosynthesis. 4. This results in overcorrections by regeneration. Reprinted by permission, with minor paraphrasing of the text, from the Croonian Lecture, "On immunity with special reference to life" delivered by Paul Ehrlich to the Royal Society, 22 March, 1900.

Figure 2. Supramolecular organization of plasma membrane and occurrence of estrogen receptors. A model of the surface membrane from an estrogen-responsive cell in the region of a caveolar structure is depicted. Estradiol may interact with one of several different forms of membrane-associated estrogen receptors (ER). The precise physical and full structural characterization of these molecules remain to be established. They may be known membrane components, such as enzymes, G-proteins, ion channels or receptors for non-steroid ligands, with previously unrecognized binding sites for steroids (1); new isoforms of steroid hormone receptors (2); 'classical' receptors complexed with other membrane-associated proteins (3); or novel membrane proteins (4). Similar to ER, androgen receptor co-localizes with caveolin-rich membrane fractions from target cells, and androgen receptor directly interacts with caveolin-1 in an androgen-dependent process, providing evidence for a potential physiological role of this interaction. Of note, alternatively-spliced transcripts of several steroid receptors occur, and these variant receptors give rise to proteins of different molecular size and, possibly, modified properties. Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions. ER-α, for example, contains several hydrophobic regions, but it is unknown whether these are sufficient for disposition as an integral membrane protein. Post-translational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation and/or addition of lipid anchors or other alterations, such as palmitoylation or myristolation.

Figure 3. Schematic representation of pathways for the internalization of extracellular agonists. Revised from Szego and Pietras (1984) and reprinted with permission.

Figure 4. Binding of fluorescein-isothiocyanate (FITC)-labeled antiserum to estradiol and isolated liver cells to estradiol immobilized by covalent linkage to albumin-derivatized nylon fibers. Incubation was conducted at 22° C. with (A) FITC-labeled non-immune serum, or (B) estrogen antiserum, the latter demonstrating availability of the steroid at the fiber surface, as shown in darkfield-UV fluorescence micrographs (x100). In independent experiments, (C) and (D), cells derived from liver

were incubated with the derivatized fibers in Ca^{2+}, Mg^{2+}-free Ringer solution. Washed fibers with bound cells were photographed with an immersion lens. Some cells appear fairly rounded, while others tend to flatten out at the fiber surface (x850). Reprinted with permission from Pietras and Szego (1979).

Figure 5. Electron microscopic visualization of receptor-mediated, specific binding and internalization of 17ß-estradiol-17-hemisuccinate:BSA that had been adsorbed to colloidal gold (E17 BSA:Au) at surfaces of human hepatoblastoma (Hep G2) cells. Note binding of ligand to the plasma membrane directly over a potential clathrin-coated endocytotic pit (arrow head) and intracellular tubulovesicular structures beneath it (small arrows). In control preparations with BSA:Au (lacking derivatization with estrogen; not shown), there is minimal internalization, despite its presence in abundant extracellular concentrations. Scale bar, 0.250 µm. Reprinted with permission from Moats and Ramirez (2000).

Figure 6. Electron microscopic demonstration of localization of immunocreactivity to peroxidase-labeled receptor for alpha isoform of estrogen receptor (ERα) in the hippocampal formation of proestrous rats. Both genomic and nongenomic functions are implicit in the distribution of immunoreactivity. A. Label is seen throughout the nucleus (N) of a neuron in the hilus of the dentate gyrus, as well as a few patches in the cytoplasm (arrowhead), and also at the plasmalemma (small arrows). B. In another cell, a dense patch of immunoreactivity is seen in the nuclear envelope, while in C, an intensely labeled endosome (En) occurs in the perinuclear cytoplasm near the Golgi apparatus (G). Additional ERα labeling was affiliated with the perikaryal plasmalemma and is apparent in dense patches of reaction product adjacent to several cytoplasmic organelles (B and C). Extranuclear sites revealed with the present methods had not been identified previously by light microscopy. Scale bars = 0.5 µm. Reprinted with permission from Milner *et al.* (2001).

Figure 7. Low-magnification electron micrographic views of luminal surfaces of uterine epithelial cells of ovariectomized rats at brief intervals after iv administration of control vehicle (A) or E_2ß, 0.5 µg/100 g body wt (B-I). Relative paucity of microvilli (MV) in control preparation is in contrast to striking onset and progressive enhancement of these structures at 35 (B), 45 (C), 80 (D) and 120 (E) sec after exposure to hormone *in vivo*. F-I: cell surfaces at 5, 10, 15, and 30 min, sequentially, reveal the remarkable subsidence of the above MV activity. Thus, by 30 min after estrogen (I), the degree of luminal surface investment with MV closely resembles the relatively quiescent control state (A). ds, Desmosomes. Reprinted by permission from Szego *et al.*, (1988).

Figure 8. Schematic representation of time course of responses of uterus to estradiol-17ß . Times shown on the logarithmic scale refer to onset of unequivocal change from baseline values. Thus, times indicated are dependent in part upon sensitivities of the various analytic methods applied and upon the somewhat arbitrary selection of initial time-points for observation in the several experimental protocols. Reprinted with permission from Szego and Pietras (1984), and amplified with further data.

Figure 9. The response as continuum: signal transduction mechanisms leading to the full sequence of receptor-mediated responses of the target cell to steroid hormone. Postulated mechanism of action of a steroid hormone (black circles) in target cells with a steroid hormone receptor (HR) is shown. Steroid ligands bind first to membrane-associated receptors (*cf.* Fig. 2). The liganded membrane receptor may affect one or more of several pathways, including phospholipase C (PLC) or protein kinase C (PKC) signaling, leading to modulation of ion channels and enhanced flux of ions, notably Ca^{++}; interaction with peptide or growth factor membrane receptors (GFR) and their immediate signaling partners (SOS, Grb, Ras); activation of MAP kinase cascades (Raf-MEK-MAPK) or G-proteins and nucleotide cyclases (AC) with generation of cyclic nucleotides (cAMP) and modulation of protein kinases (PKA). These primary membrane interactions may promote physical alteration of the steroid receptor itself, such as phosphorylation, via steroid-induced or ligand-independent pathways. In some cases, steroid receptors then associate with vesicular structures and microtubule-microfilament (mf) elements in the cell interior and gain access to other subcellular compartments. Liganded steroid receptor in the nucleus may promote association of the receptor with coactivator proteins and with specific hormone-responsive elements (HRE) in DNA, leading, in turn, to initiation of selective gene transcription. The wide array of cell responses to steroid hormones may occur as a consequence of synergistic feed-forward circuits where steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of specific receptors in the nucleus.

TABLE I

General, Receptor-Mediated Functions of the Steroid Hormone Super-Family

Estrogen	Growth and development of reproductive targets, including breast, bone, liver and cardiovascular system
Androgen	Reproductive tract functions, patterns of hair growth, influences on brain and libido in both sexes
Progesterone	Components of reproductive function and behavior, meiosis in oocytes, acrosome reaction in sperm
Glucocorticoids	Maintenance of integrity of cell membranes; metabolic functions in protein mobilization and gluconeogenesis; neurone signaling; immune and inflammatory reactions; apoptosis
Aldosterone	Promotion of reabsorption of sodium and excretion of potassium in kidney, colon, urinary bladder; acute effects on cardiac function and on sodium transport in smooth muscle
Digitalis-like	Inotropic and chronotropic effects on heart; inhibition of Na^+,K^+-ATPase in this and many other tissues
Vitamin D	Regulation of Ca^{2+} and phosphate homeostasis; promotion of differentiation of many cell types
Retinoids	Control of cell growth during embryonic development; anti-oxidant function promotes integrity of epithelial and many other tissues
Thyroid hormone	Energy expenditure; embryonic development and postnatal maturation of various tissues, including bone and brain

TABLE II

Parallels in Membrane-Initiated Phenomena Induced by Glucocorticoids and Vitamin D Metabolites

TIME	GLUCOCORTICOIDS	$1,25(OH)_2D_3$
Seconds	Binding to surface receptor	Binding to surface receptor
	Electrophysiological effects	Ca^{2+} channel activation
	Binding to intracellular receptors	Membrane receptor internalization
Minutes	PKC activation/translocation[1]	PKC activation/translocation
		PKA activation
	Capping of membrane receptors[2]	Vesicular loading of P_i, Ca^{2+}
	Decreased P_i uptake[1]	Increased P_i Ca^{2+}
transport[3]		
		Secretion of calbindin,cathepsin B[3]
		Phosphorylation of osteopontin[4]
Hours	Enzyme synthesis[5]	Synthesis of Ca^{2+} binding proteins
		Synthesis of α-tubulin[3]
		Proliferation of lysosomes[3]
	Apoptosis[2]	Cell differentiation/migration

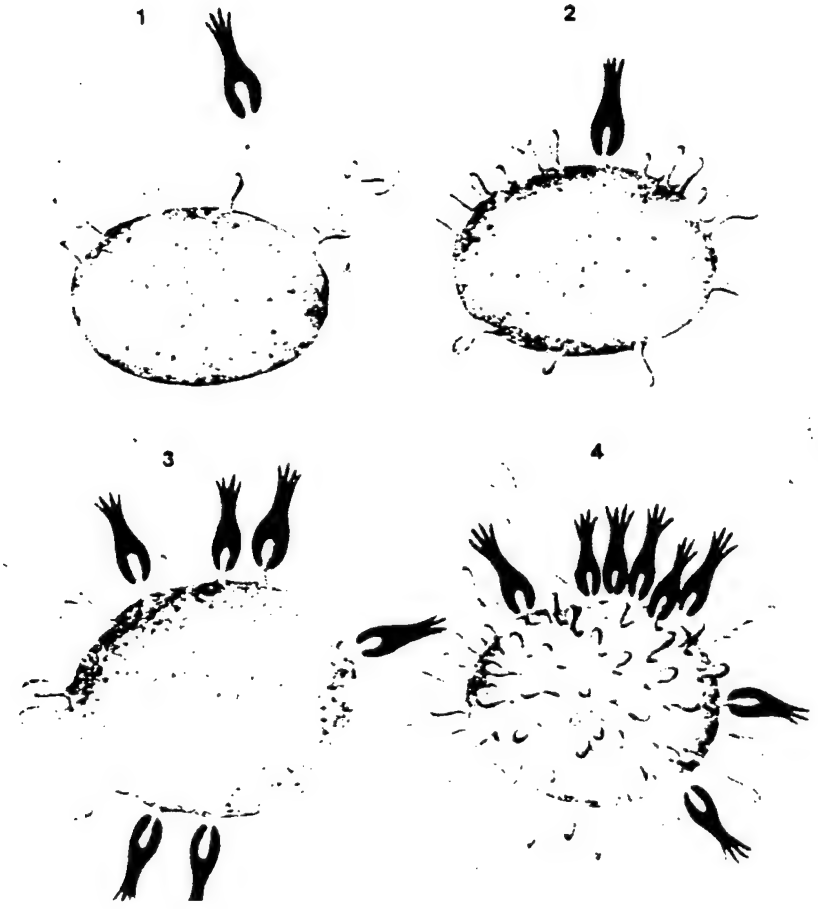
[1]Kidney; [2]lymphocytes; [3]intestine; [4]bone, [5]liver

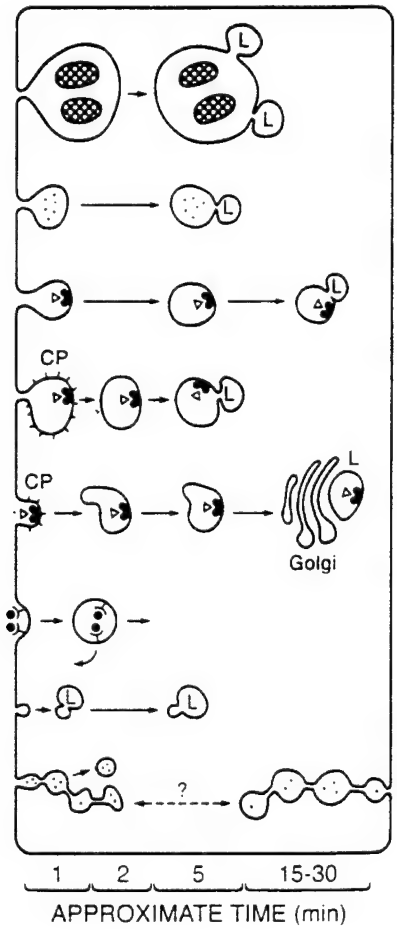
TABLE III

Examples of Acute, Receptor-Mediated Signals of Plasma Membrane Perturbation*

- Alterations in Na^+, K^+-ATPase activity
- Rapid shifts in availability of cyclic nucleotides
- Fluxes in Ca^{2+} and other ions, with potential for modulation of neural activities and numerous enzymatic and mechanoeffector systems
- Activation of the phosphoinositide cascade
- Release of endogenous amines and nitric oxide, with influence on microcirculation
- Structural reorganization of the cell surface, with potential for intracytoplasmic communication; formation of endosomes
- Accentuated delivery, in microquanta, of components of lysosomes to the cell surface and interior

* Properties shared, to various degrees, by steroid and peptide hormones, as well as many other effectors, including neurotransmitters, lectins, and toxins


1
2
3
4



PHAGOCYTOSIS: VACUOLES.
dia >1um [Particles]

MACROPINOCYTOSIS: VESICLES.
300-1000nm [Ferritin]

RECEPTOR-MEDIATED MACRO-
PINOCYTOSIS: [HLA-Antigen]

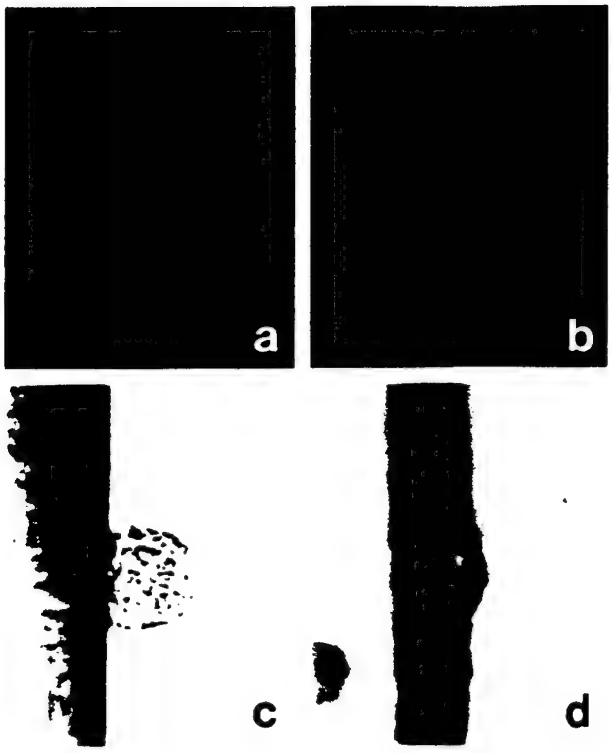
RECEPTOR-MEDIATED PINOCYTOSIS
VIA COATED PIT: 50-250nm
[LDL-cholesterol]

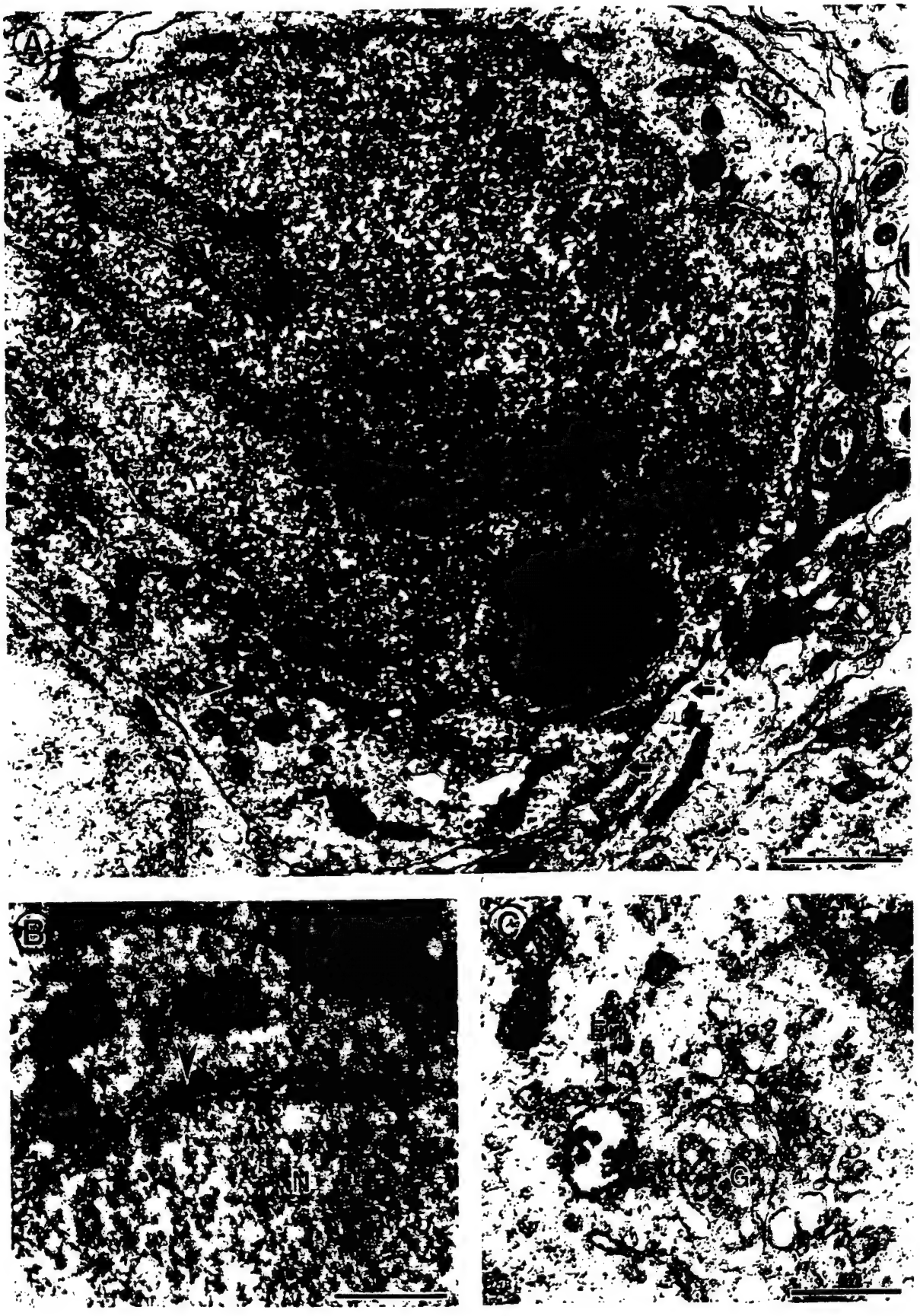
RECEPTOR-MEDIATED PINOCYTOSIS
VIA COATED PIT & 'RECEPTOSOME':
150-350nm [α_2-MG]

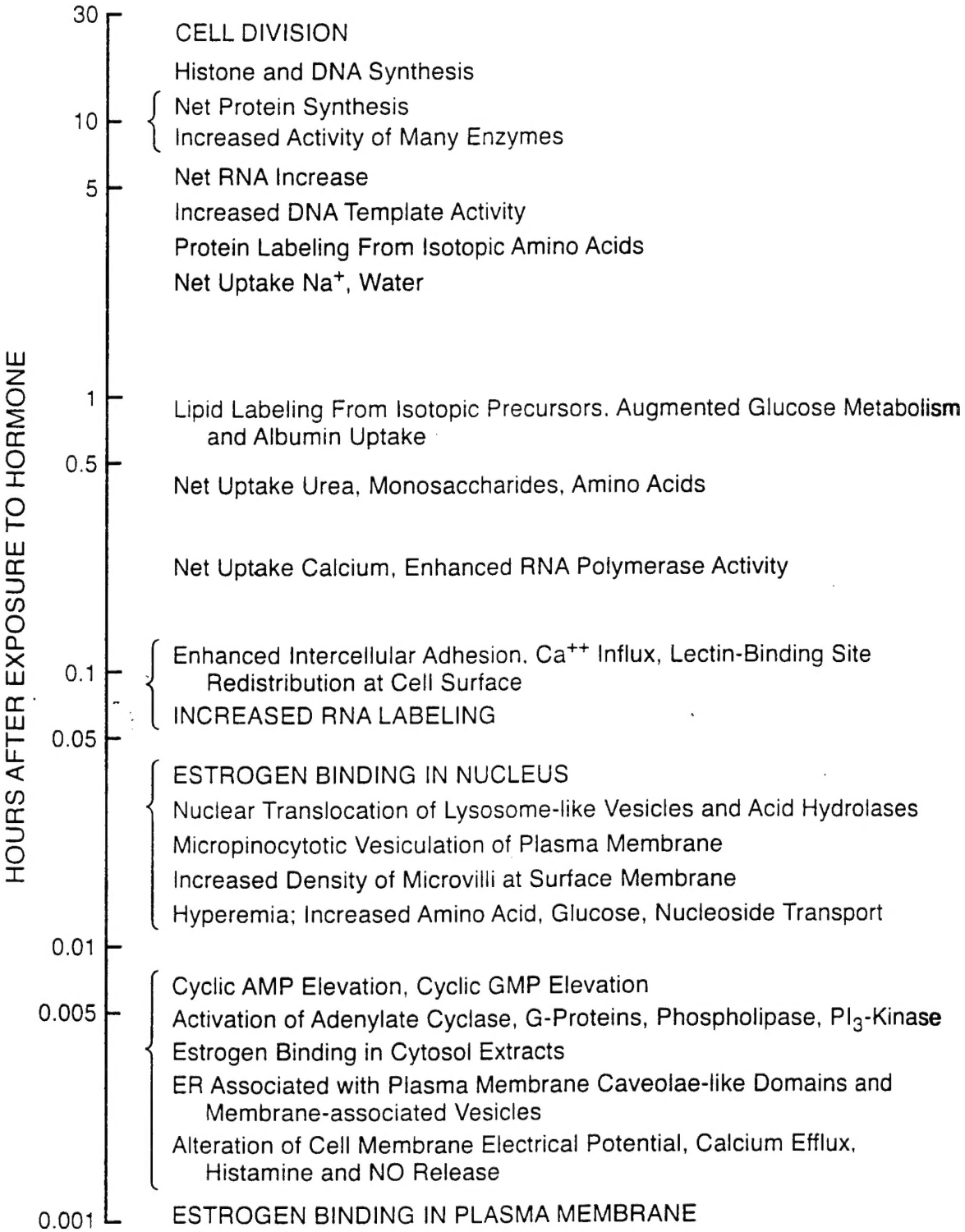
RECEPTOR-MEDIATED ENDOCYTOSIS
VIA CAVEOLAE: 50-100nm

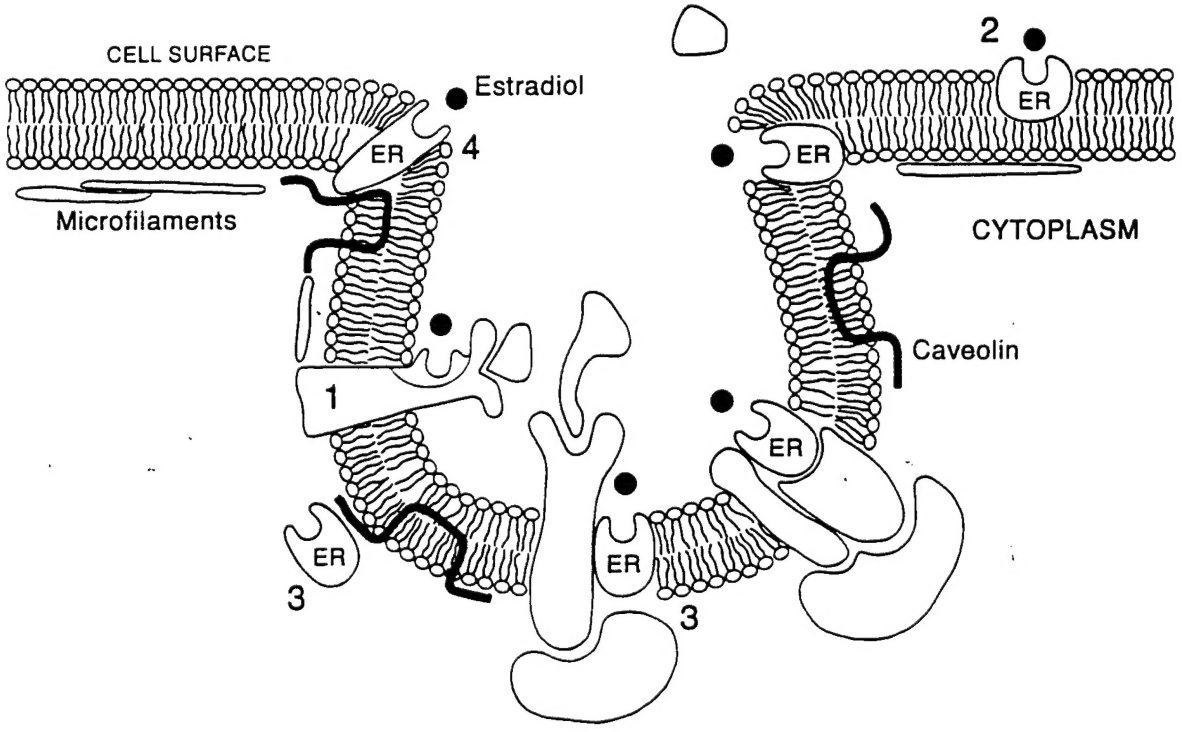
MICROPINOCYTOSIS: 70nm

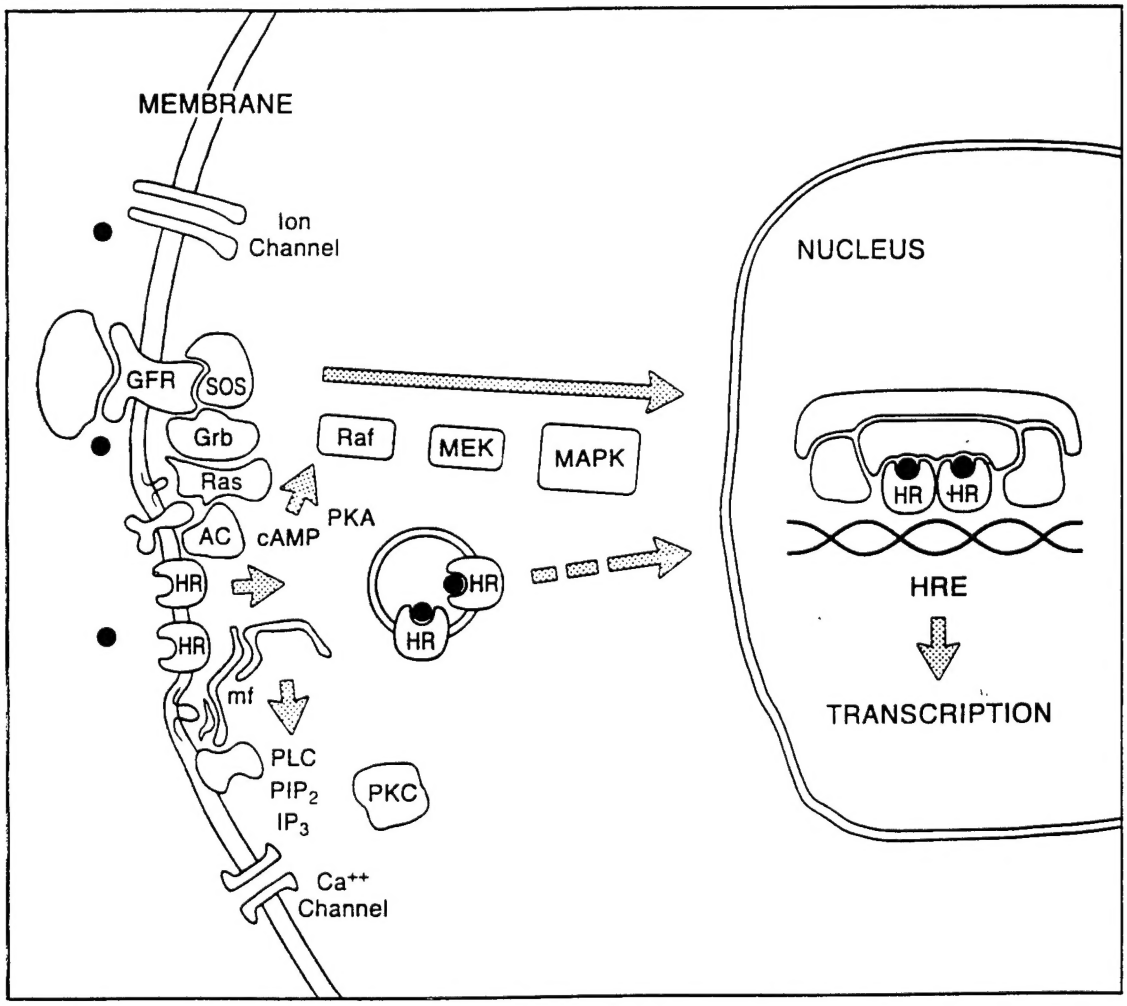
RACEMOSE VESICULATION:
50-200nm: DIFFUSION


a
b
c
d


A
B
C
G


HOURS AFTER EXPOSURE TO HORMONE
30
10
5
1
0.5
0.1
0.05
0.01
0.005
0.001
CELL DIVISION
Histone and DNA Synthesis
Net Protein Synthesis
Increased Activity of Many Enzymes
Net RNA Increase
Increased DNA Template Activity
Protein Labeling From Isotopic Amino Acids
Net Uptake Na+, Water
Lipid Labeling From Isotopic Precursors. Augmented Glucose Metabolism and Albumin Uptake
Net Uptake Urea, Monosaccharides, Amino Acids
Net Uptake Calcium, Enhanced RNA Polymerase Activity
Enhanced Intercellular Adhesion. Ca++ Influx, Lectin-Binding Site Redistribution at Cell Surface
INCREASED RNA LABELING
ESTROGEN BINDING IN NUCLEUS
Nuclear Translocation of Lysosome-like Vesicles and Acid Hydrolases
Micropinocytotic Vesiculation of Plasma Membrane
Increased Density of Microvilli at Surface Membrane
Hyperemia; Increased Amino Acid, Glucose, Nucleoside Transport
Cyclic AMP Elevation, Cyclic GMP Elevation
Activation of Adenylate Cyclase, G-Proteins, Phospholipase, PI3-Kinase
Estrogen Binding in Cytosol Extracts
ER Associated with Plasma Membrane Caveolae-like Domains and Membrane-associated Vesicles
Alteration of Cell Membrane Electrical Potential, Calcium Efflux, Histamine and NO Release
ESTROGEN BINDING IN PLASMA MEMBRANE


CELL SURFACE
Estradiol
ER
4
Microfilaments
2
ER
ER
CYTOPLASM
Caveolin
1
ER
ER
3
ER
3


MEMBRANE
Ion
Channel
GFR
SOS
Grb
Ras
AC
cAMP
PKA
HR
HR
mf
PLC
PIP2
IP3
PKC
Ca++
Channel
Raf
MEK
MAPK
HR
HR
HR
NUCLEUS
HR
HR
HRE
TRANSCRIPTION